Immunity of the Buffalo Mammary Gland during Different Physiological Stages

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ABSTRACT : To study the immunity of the buffalo mammary gland during involution and around parturition and compare it with the mastitic mammary gland, milk samples were collected from 9 Murrah buffaloes during the above critical periods. SCC of buffalo milk increased significantly ($p<0.01$) by day 21 of involution and one week prepartum. SCC was significantly higher around parturition but became normal at 14 days postpartum. Phagocytic activity (PA) and phagocytic index (PI) of the buffalo milk neutrophils decreased as the duration of the dry period increased. Elevated levels of immunoglobulins at calving improved the PA and PI, but the lowest PA of 18.8% and PI of 1.75 were recorded at 7 days postpartum. Buffaloes suffering from clinical mastitis had PA of 12.3% and PI of 1.46 that increased significantly ($p<0.01$) on the third day of treatment. Distance of teat from ground level was found to be minimum at one week before parturition. The investigation showed that *in vitro* phagocytic activity of buffalo neutrophils was weakest at one week postpartum. (**Key Words :** Buffalo, Milk, SCC, Phagocytic Activity, Index, Immunoglobulins, Teat Distance)

INTRODUCTION

The dry period and the period soon after parturition are generally accepted as the most critical periods with respect to mammary health in a dairy cow. During this period the mammary gland undergoes marked biochemical, cellular and immunomodulatory changes to accommodate involution, to prepare for parturition, to withstand the stress of parturition, to transform colostrum into milk and then for the attainment of peak milk production. Additionally, the early dry period and periparturient period have been promoted as the times of highest incidence for new intramammary infections and the first 30 days of lactation has been reported to be the time of highest incidence of clinical mastitis in cows (Erskine, 2001). To counter any bacterial infection PMN (polymorphonuclear neutrophils) constitute the first line of defence. It is the rapid recruitment of sufficient numbers of PMN into the mammary gland and increased phagocytosis at the beginning of infection that prevents establishment of mastitis (Paape et al., 2003). *In vitro* analysis of neutrophil function provides a very effective tool for the study of natural mastitis resistance in cows (Macdonald et al., 1994). In buffaloes, which are the major contributors of milk production in South Asia, there is no study which records the immunity of the buffalo

mammary gland during the above-mentioned critical period. Therefore, the present study was proposed to estimate the defensive ability of the mammary gland during involution and after parturition and compared them with the changes undergoing in the mastitic mammary gland of buffaloes.

MATERIALS AND METHODS

Selection of animals

Nine dry and pregnant Murrah buffaloes were selected from the Institute's herd. All the animals were kept in a loose housing system with brick flooring and were managed as per the practices followed in the institute's herd. The animals were offered *ad libitum* green fodder and a calculated amount of concentrate mixture based on milk production was offered only at the time of milking. Fresh tap water was available *ad libitum* at all times of the day. Milk samples were collected from these buffaloes one week before drying (-7), on the day of drying (day 1), during active involution, i.e. days 7, 14 and 21 of drying, one week before parturition and after parturition i.e. days 0, 1, 7 and 14 postpartum. To compare the immunity of infected mammary gland with involuting and newly calved buffaloes, milk samples were also collected from 9 clinically mastitic buffaloes which were reported for mastitis in the Animal Health Complex on day 1 and 3 of mastitis. Distances of anterior and posterior teats from the ground were measured with the help of a cloth tape during all the above periods.

Total milk immunoglobulins were estimated in all the samples by the method of McEwan et al. (1970).

In vitro phagocytosis assay

Milk samples were collected from the normal and mastitic quarters of Murrah buffaloes and filtered separately through a nylon filter (40 μm pore size) and diluted to 60% with cold Dulbecco's PBS (volume/volume). Isolation of PMN from milk was performed as described by Mehrzad et al. (2001). *In vitro* phagocytosis assay was performed as described by Hay and Westwood (2002). Yeast cells were used for determining phagocytosis. Yeast (*Saccharomyces cerevisiae*) was obtained from the National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal. Yeast cells were maintained by sub-culturing fortnightly in potato dextrose broth and incubated at 30°C for 48 h and stored under refrigeration between transfers.

Yeast cells were cultured in potato dextrose broth for 48 h at 30°C and autoclaved at 120°C for 45 min in culture medium and then washed three times with phosphate buffer saline (PBS). Different aliquots were stored at 4°C until use. Just before use, yeast cells were sonicated gently to disrupt clumps and diluted to 10^8/ml in DMEM Ham's F-12 medium (with phenol red). DMEM Ham's F-12 medium was prepared from DMEM medium (1.56 g/L), NaHCO₃ (0.12 gm/100 ml), bovine serum albumin (0.1%), streptomycin (50 μg/ml) and penicillin-G (200 IU/ml), and the pH was adjusted to 7.2.

One ml of neutrophil suspension (1×10^6 cells/ml) was added to a 35 mm Petri dish and incubated at 37°C for 2 h followed by washing with medium. After washing, 1 ml of medium was added and further incubation for 2 h at 37°C was carried out. 100 μl yeast cell suspension (10^8 cells/ml) was added to the medium and incubated for 1 h at 37°C in a 5% CO_2 humidified incubator followed by washing twice gently with culture medium and addition of 1 ml of 1% tannic acid solution for 1 min. It was again washed with medium and dried in air. Staining was done with May-Grunwald stain freshly diluted with Giemsa buffer (1:2) for 5 min. (Giemsa buffer was prepared by weighing 2.1 g/100 ml citric acid and Na₂HPO₄ 2.83 g/100 ml: 8.5 ml of citric acid was mixed with 11.5 ml Na₂HPO₄ and the pH was adjusted to 5.75 and made to a volume of 100 ml). After washing with buffer it was air-dried. The culture was again stained with Giemsa solution, freshly diluted with buffer (1:2) for 15 min. and washed with buffer and air-dried. Observations were taken at 1,000×magnification under oil immersion (Olympus, BX 50 Microscope).

Calculation of phagocytic activity of neutrophils

Percentage phagocytosing neutrophils was recorded as percentage activity (PA) and average number of yeast cells per neutrophil was recorded as mean phagocytosis or phagocytic index (PI) as described by Guidry et al. (1976).

Direct microscopic somatic cell count

SCC of each original milk sample was determined in duplicate within 6-h post collection. The milk was heated to 40°C in a water-bath and held for 15 min at that temperature before being cooled to 20°C with careful stirring. 0.01 ml of milk was spread on a 1-cm^2 (0.5×2 cm) area of a degreased microscopic slide and was dried in a horizontal position. SCC of milk samples were measured microscopically by the method of Gonzalo et al. (2003). Only those cells which possessed a stained nucleus were counted. Differential cell counting (DLC) was also carried out to determine the presence of different cell types like epithelial cells, lymphocytes, neutrophils and macrophages in milk. For viewing and differentiating various cells the focus was fixed and the microscope's fine focus was constantly adjusted. Milk neutrophils had a multilobed nucleus with bridges, milk lymphocytes were distinguished by having a deeply staining nucleus, which may be eccentric in location and have a relatively small amount of cytoplasm. Milk macrophages were identified as the largest cell type seen in milk, whereas, epithelial cells were smaller and had a spherical nucleus. The somatic cell counts were measured under the microscope with a magnification of 400× in 50 fields and average number of cells per field was multiplied by the microscopic factor (0.882). The microscopic factor was determined by using an occular and stage micrometer. Somatic cell counts/ml of milk (lakh) = average cells count in one field ×0.882.

Statistical analysis

The results were expressed as mean±standard error of mean. Significance was tested by employing analysis of variance (ANOVA) and comparison between means was made by critical difference (CD) value.

RESULTS AND DISCUSSION

The present study was conducted for the first time to study the changes in the immunity of buffalo mammary gland during three physiological stages i.e. during involution, two weeks postpartum and during mastitis. The results obtained under different physiological stages have been presented separately under various headings below:

In this study, milk neutrophils were challenged with particles of *S. cerevisiae* (zymosan) because these particles are easily recognizable in light and electron microscopic analysis and are not capable of invading cells, thus assuring that the internalized particles result from phagocytosis (Amarante-Paffaro et al., 2004). Further, there was no difficulty in differentiating between ingestion and the

Table 1. Milk SCC, DLC and total immunoglobulins during involution of buffalo mammary gland

	Days dry					
	-7	1	7	14	21	40
SCC ($\times 10^5$)	$1.42^a \pm 0.07$	$1.34^a \pm 0.05$	$3.38^a \pm 0.24$	$3.45^d \pm 0.14$	$12.92^b \pm 0.96$	$15.87^c \pm 1.56$
Epithelial cells ($\times 10^5$)	$0.96^c \pm 0.06$	$0.92^c \pm 0.05$	$1.93^d \pm 0.98$	$0.92^c \pm 0.04$	$0.66^b \pm 0.08$	$0.18^a \pm 0.02$
WBC ($\times 10^5$)	$0.46^a \pm 0.02$	$0.42^a \pm 0.01$	$1.44^a \pm 0.15$	$2.53^a \pm 0.13$	$12.25^b \pm 0.89$	$15.68^c \pm 1.54$
Neutrophils (%)	$18.55^b \pm 1.29$	$18.66^b \pm 1.56$	$23.56^c \pm 0.80$	$17.77^b \pm 1.07$	$14.00^b \pm 1.33$	8.50 ± 2.04
Macrophages (%)	$36.22^a \pm 1.41$	$36.11^a \pm 1.00$	$37.55^a \pm 1.13$	$41.33^a \pm 0.98$	$35.77^a \pm 6.55$	$36.00^a \pm 4.37$
Lymphocytes (%)	$45.11^{ab} \pm 2.09$	$45.22^{ab} \pm 2.06$	$38.55^a \pm 1.16$	$40.44^a \pm 0.88$	$50.11^b \pm 5.56$	$55.50^b \pm 3.40$
Total IgGs (%)	$1.51^a \pm 0.11$	$1.42^a \pm 0.10$	$1.84^a \pm 0.08$	$2.68^b \pm 0.14$	$3.11^{bc} \pm 0.21$	$3.66^c \pm 0.37$

SCC = Somatic cell counts, WBC = White blood cells.
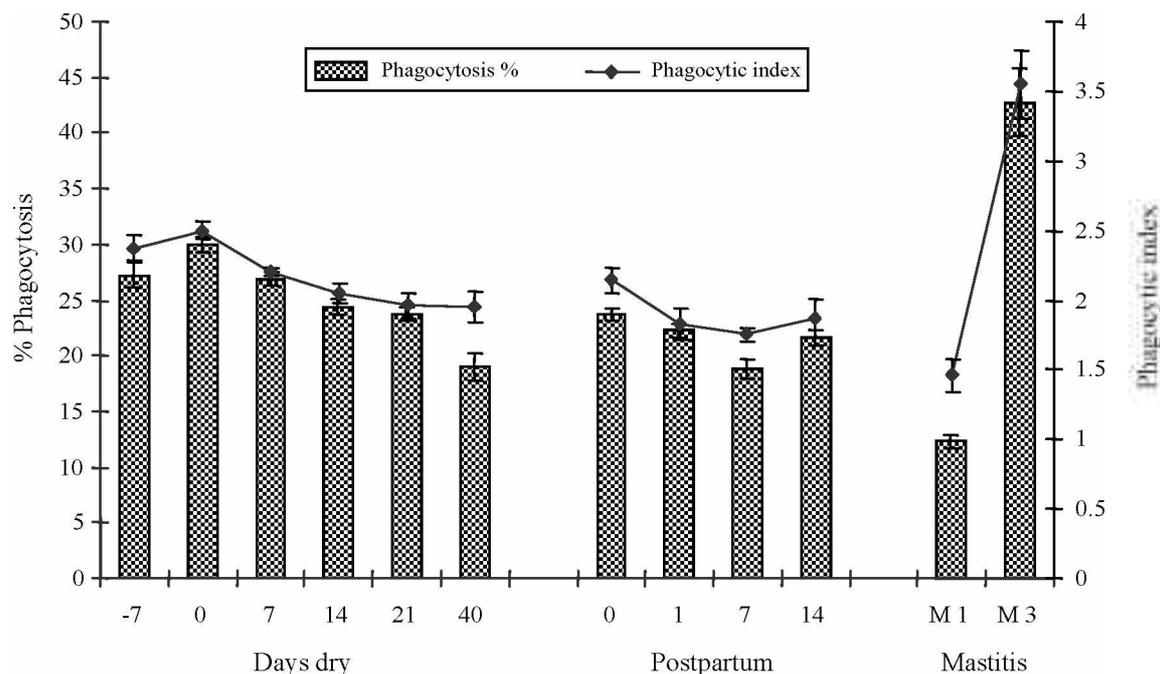
Figure 1. Phagocytic activity and index in milk of Murrah buffaloes during different physiological stages.

binding of microorganisms to the cell surface as reported by Schuit (1979) in plasma neutrophils.

During mammary involution

The results of milk SCC, DLC and total milk IgG are presented in Table 1. Values of SCC in milk of Murrah buffaloes one week before lactation were significantly less (p<0.01) than that reported in Mediterranean buffaloes (Tripaldi et al., 2003). During first two weeks of the dry period, milk SCC of the dry buffalo mammary gland increased non-significantly. A significant increase (p<0.01) in milk SCC was observed during the third week and one week before parturition. Epithelial cells increased significantly during the first week of involution and than decreased significantly (p<0.01) during different weeks of involution. During the first two weeks of the dry period, milk leucocytes increased non-significantly; a significant increase (p<0.01) was observed during the third week and one week before parturition. Milk neutrophils increased

significantly during the first week of involution. During the 2nd, 3rd and one week before involution neutrophils decreased significantly (p<0.01). A non-significant change was observed in milk macrophages of buffalo during different days of involution. Milk lymphocytes decreased during the first week of involution and than increased significantly (p<0.01) during different weeks of involution. Total immunoglobulins increased during the different weeks of drying, but the changes were non-significant. These changes occur to prepare the mammary gland for involution during the early dry period (Jensen and Eberhart, 1981). The functional role of the phagocytic cells (neutrophils and macrophages) during early involution is generally thought to be that of phagocytosis of degenerated secretory epithelial cells, fat and casein (Smith and Hogan, 2001).

Changes in the phagocytic activity and distance of teats from the ground level during prepartum, postpartum and during mastitis are presented in Figure 1 and 2. Percent phagocytosis decreased during the first week of active
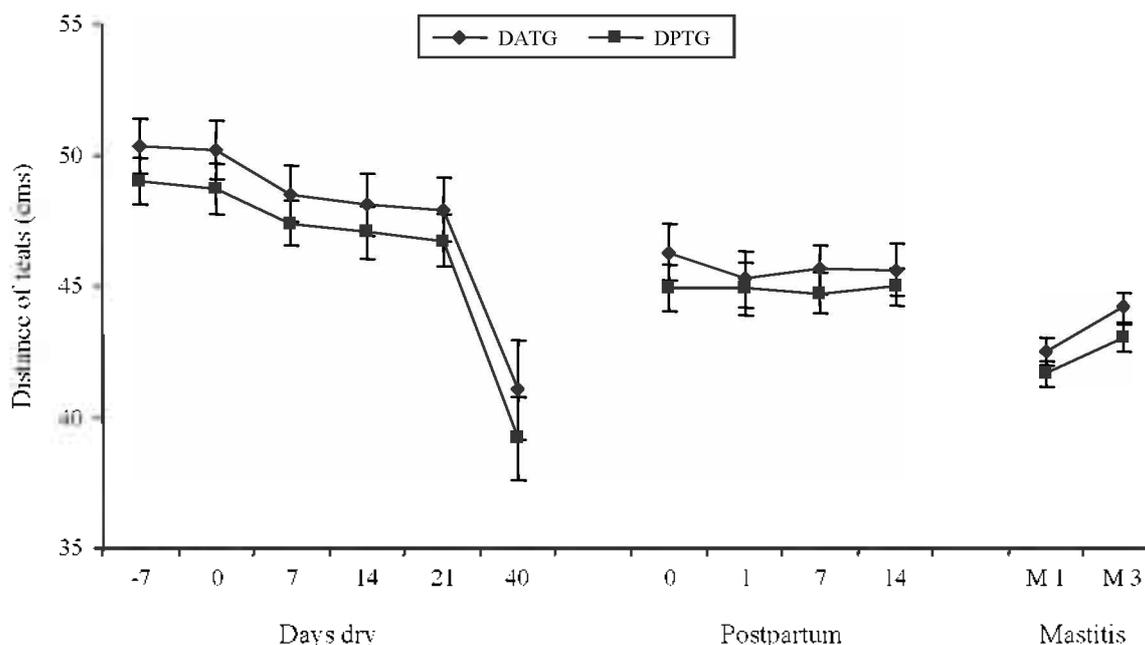
Figure 2. Distance of anterior teats (DATG) and posterior teats (DTPG) from ground level in Murrah buffaloes during different physiological stages.

Table 2. Milk SCC, DLC and total immunoglobulins during the postpartum period

	Days postpartum			
	0	1	7	14
SCC ($\times 10^5$)	$4.70^b \pm 0.46$	$3.54^b \pm 0.40$	$2.27^a \pm 0.15$	$1.23^a \pm 0.07$
Epithelial cells ($\times 10^5$)	$1.69^{ab} \pm 0.19$	$2.18^c \pm 0.33$	$1.24^d \pm 0.07$	$0.85^d \pm 0.05$
WBC ($\times 10^5$)	$3.00^c \pm 0.29$	$1.35^b \pm 0.12$	$1.02^b \pm 0.08$	$0.37^a \pm 0.02$
Neutrophils (%)	$16.11^a \pm 1.64$	$22.22^a \pm 1.26$	$20.66^a \pm 2.27$	$17.44^a \pm 1.48$
Macrophages (%)	$46.88^b \pm 2.93$	$44.11^{ab} \pm 2.63$	$37.55^a \pm 3.18$	$34.66^a \pm 2.92$
Lymphocytes (%)	$36.77^{ab} \pm 2.17$	$33.66^a \pm 1.92$	$41.55^{ab} \pm 3.35$	$46.22^b \pm 2.67$
Total IgGs (%)	$5.71^c \pm 0.14$	$3.53^b \pm 0.23$	$1.24^a \pm 0.07$	$1.44^a \pm 0.10$

SCC = Somatic cell counts, WBC = White blood cells.

involution, became constant during the 2nd week and then again decreased during the third week and one week before parturition. Phagocytic index also decreased during the first week, again decreased during the 2nd and 3rd week and than significantly decreased (p<0.01) during the 3rd week of drying. However, no difference in the phagocytic index was observed in the milk samples collected one week before parturition.

During the first week of involution, *in vivo* neutrophils are occupied in the phagocytosis of degenerated epithelial cells, fat and casein particles, thus limiting their ability to phagocyte pathogens. But, phagocytic activity *in vitro* of isolated milk neutrophils during this period was also low (Paape et al., 2003). There is reason to suspect that there are factors (other than fat and casein) present in the secretion of the involuting gland that may reduce the phagocytic capabilities of the macrophage and PMN and reduce the responsiveness of lymphocytes to antigen stimulation (Smith and Hogan, 2001). Immunoglobulins and serum albumin tended to increase during the 1st week of involution

due to break down of tight junctions. The magnitude of increase in selective transfer is far less than that observed during colostrogenesis. The transitory nature of the increase may suggest that receptor sites required for selective transfer are either temporarily synthesized in greater numbers or made accessible as a result of the early involution events occurring in the secretory epithelial cell (Smith and Hogan, 2001).

With the cessation of periodic milk removal, milk accumulates in the dry mammary gland and its distance from ground level decreases. Distance of anterior and posterior teats from the ground showed a non-significant change during the period of active involution, however a significant decrease was observed during one week before parturition.

During the dry period, SCC was found to be negatively correlated with epithelial cells. PA and PI SCC was positively correlated with total milk leucocytes and immunoglobulins. Neutrophils were negatively correlated with lymphocytes and total IgG but positively correlated

Table 3. Milk SCC, DLC and total immunoglobulins during mastitis

	During mastitis	
	M 1	M 3
SCC ($\times 10^5$)	$34.82^b \pm 5.51$	$17.69^a \pm 2.44$
Epithelial cells ($\times 10^5$)	$7.10^b \pm 0.98$	$3.92^a \pm 0.51$
WBC ($\times 10^5$)	$27.74^b \pm 4.53$	$13.76^a \pm 1.94$
Neutrophils (%)	$70.66^a \pm 7.43$	$63.82^a \pm 2.65$
Macrophages (%)	$14.44^a \pm 1.00$	$22.34^b \pm 1.57$
Lymphocytes (%)	$2.33^a \pm 0.57$	$11.72^b \pm 1.76$
Total IgGs (%)	$1.15^a \pm 0.05$	$1.17^a \pm 0.06$

SCC = Somatic cell counts, WBC = White blood cells.
The values with same symbols are statistically at par within a row whereas with different symbols are significantly different at $p<0.01$.

with PA and PI. Distance of both anterior and posterior teats from ground level were also found to be negatively correlated with total IgG.

During postpartum

The results of milk SCC, DLC and total milk IgG are presented in Table 2. On the day of calving the values of buffalo milk SCC were 4.7 lakhs per ml of milk. These values decreased significantly after the first week. Milk epithelial cells increased on day 1 of calving and then decreased on days 7 and 14 of calving. Milk leucocytes decreased significantly on day 1 of calving and again on day 14 after calving. A non-significant increase was observed in milk neutrophils during different days of postpartum. Macrophages decreased significantly on day 1 of calving and than changed non-significantly. Milk lymphocytes first decreased significantly and then increased significantly ($p<0.01$) during the 1st and 2nd week after calving. Total immunoglobulins were high on the day of calving and than decreased afterwards. Percent phagocytosis was high on day 0 and 1 of calving, decreased significantly on day 7 and then reached the day 1 levels on day 14 after calving. A non-significant change was observed in the distance of teats from the ground level during the postpartum period.

Lactation stage affects the SCC so that immediately after parturition SCC is high, but decreases rapidly to the normal level within 4-5 days after calving (Barkema et al., 1999). Lymphocytes were the most prominent cells followed by macrophages and neutrophils; however. Jensen and Eberhart (1981) reported an increase in neutrophils and a decrease in lymphocytes as parturition approached in HF cows. A phagocytosis promoting effect was found in colostrum on day 0, followed by day 1, with maximum activity obtained at parturition which gradually decreased with the time course of lactation as well as IgG level. Sugisawa et al. (2001) found that as the levels of immunoglobulins were very high in colostrum, PA was increased by up to 25% and was dose dependent. The

phagocytosis activity then decreased during day I of calving and was minimum on day 7 after parturition. The respiratory burst activity of milk PMN is impaired during early lactation because myeloperoxidase-catalyzed bactericidal activity of milk cells is impaired during early lactation (De-Chatelet et al., 1982). Reduction in phagocytic activity of milk neutrophils during the early lactation period may be due to hormonal and metabolic changes such as glucocorticoids, ketone bodies and pregnancy associated glycoproteins (Hoeben et al., 2000). There is a general immunosuppression known to exist around parturition, which inhibits the phagocytic ability of PMN present in the secretion of the prepartum gland. Mehrzad et al. (2001) also found that the chemiluminescence response and viability of milk neutrophils were lowest between 3 d and 11 d postpartum.

Hill et al. (1979) reported that newly calved dairy cows had more severe cases of coliform mastitis than cows later in lactation due to slower migration of neutrophils into the gland. During calving, cortisol rises markedly and may have a deleterious effect on mammary immunity near calving. Leukocyte function is significantly altered in cattle challenged with dexamethasone (a synthetic glucocorticoid), and when dexamethasone was administered to lactating cows with subclinical infections, increased bacterial shedding led to the development of clinical mastitis (Lohuis, 1988). Also, important phagocyte adhesion molecules that are essential for trafficking into inflammatory sites are down-regulated by dexamethasone (Burton, 1995). Meglia (2001) found that the weeks just before and after parturition were characterised by neutrophilia, eosinopenia, lymphopenia and monocytosis, but time had no effect on neutrophil phagocytosis and oxidative burst.

During the postpartum period SCC were found to be positively correlated with epithelial cells, WBC and total immunoglobulins. Milk macrophages were found to be negatively correlated with lymphocytes but positively correlated with total IgG. PA was found to be positively correlated with PI and total IgG.

During mastitis

The results of milk SCC, DLC and total milk IgG of mastitic buffaloes are presented in Table 3. During day 1 of mastitis, milk epithelial cells and leucocytes were very high and than decreased significantly by the 3rd day of mastitis. Milk macrophages and lymphocytes decreased significantly by day 3 of mastitis, whereas, milk neutrophils decreased non-significantly. Total milk immunoglobulins changed non-significantly during the days of mastitis. Percent phagocytosis and PI was very low during day 1, but then increased significantly ($p<0.01$) on day 3 of mastitis. Although the distance of both anterior and posterior teats from ground level increased by the 3rd day of mastitis, as

the swelling of the udder decreased with treatment, but the changes were non-significant.

Mastitis is characterised by very high milk SCC and neutrophils are the first cells to migrate from blood into an inflamed area after initiation of inflammation (Jain, 1993). The main function of neutrophils is phagocytosis and intracellular killing of engulfed bacteria by two distinct mechanisms, the respiratory burst and digestion by lysosomal enzymes (Woessner, 1992). In the infected quarter of mastitic buffaloes, the neutrophils increased to 74% on day 1, which was lesser than 90% reported in cows (Pyorala, 2003). On the 3rd day in buffaloes there was a significant reduction (p<0.01) in influx of neutrophils from blood into milk, but it was still about four times more than that of normal quarters. Although the milk after 3 days of treatment appeared normal the somatic cell counts remained very high (p<0.01). It might take days, weeks, or longer for SCC to decrease even after the pathogens have been eliminated from the gland (Schultz, 1977). An increase in percentage of lymphocytes, followed by macrophages and neutrophils, found in normal mammary quarters of buffaloes corresponds well within published values (Dosogne et al., 2003). However, there are also reports of macrophages having the maximum percentage in normal milk SCC (Burvenich et al., 2003).

In the present study, increased values of SCC (p<0.01) in mastitic milk of buffaloes indicated more sloughing off of epithelial cells and migration of leucocytes from blood into milk. The stress of disease may be responsible for this. A combination of infected udders and traumatic inflammation induced by stress had a marked and potentially economic influence on SCC level (Coulon et al., 1988). However, the values of SCC of normal quarters of buffaloes were within range (Moroni et al., 2006).

PA by neutrophils isolated from mastitic milk on day 0 (before the start of treatment) was only 12%, which increased to 42% on the 3rd day of treatment. The PA of the 3rd day sample was significantly higher than normal milk because the buffaloes were recovering from mastitis after getting antibiotic treatment. Also mastitic milk is superior to normal milk, as it contains serum opsonins and complements which facilitates phagocytosis (Jain and Lasmanis, 1978). The complement system plays an important part in the innate immunity against microorganisms through its bactericidal, opsonic and phlogistic functions. The amount of complement in the milk of healthy glands of dairy cows is low. Moreover, the classical pathway of activation is not functional because of a shortage of C1q (Rainard and Riollet, 2003). When inflammation develops, the blood-derived complement components overcome the inhibitions and complement-dependent bactericidal, opsonic and phlogistic activities may be high in milk. Rivas et al. (2002) assessed the

number and function of bovine mammary-gland phagocytes and found that cows showing ≤72.3% phagocytes by cytology were regarded as non-mastitic and those showing ≥80.7% phagocytes were considered to be mastitic.

A significant relationship exists between udder size and mastitis incidence (Monrades et al., 1990; Lund et al., 1994). Distance of the infected quarter decreased from the ground level due to inflammation and swelling. A little increase in the level of immunoglobulins was due to passing of serum albumin/immunoglobulins and other serum proteins into the milk (Haenlein et al., 1973).

During mastitis, SCC were found to be positively correlated with both epithelial cells and total leucocytes but negatively correlated with lymphocytes. Milk macrophages were found to be positively correlated with lymphocytes.

Although the phagocytic ability of the milk neutrophils and killing in the mammary gland is lower than in other tissues, it remains the most critical defense once the teat end barrier is breached. Comparison of the migration of neutrophils and the phagocytic activity during different physiological stages of the buffalo mammary gland showed that there is maximum migration of neutrophils during mastitis followed by the dry period. In contrast, the newly calved mammary gland did not show any significant change. However, consideration of only the concentration of neutrophils is not sufficient to predict phagocytosis activity during any particular period, even though there is neutrophil migration during both involution and mastitis. The phagocytic activity of mastitic neutrophils is high because the blood-mammary barrier becomes more porous during mastitis and the neutrophils get activated by the complement system/antibodies made available to them from blood. This activation increases the bactericidal activity by opsonizing the bacteria and neutralizing their toxins thus improving the overall phagocytic activity. On the other hand, neutrophils obtained from the milk of newly calved buffaloes, despite their low concentration, have to bear the effect of general immunosuppression prevalent around parturition, which inhibits their phagocytic activity.

Results of the above study indicate that, as in cows, the change in buffalo milk SCC and immunity of the mammary gland is disturbed during both involution and postpartum. Although the incidence of mastitis in buffaloes is less, their response to an infection is similar to that of cows. Buffaloes possess a powerful defence mechanism against mastitis due to their tight teat sphincter (Hogberg and Lind, 2003) and long narrow teat canal, which can be expected to effectively prevent micro-organisms from invading the udder (Uppal et al., 1994). Therefore, the chances of infection during the dry period are low. But once the buffaloes are milked after parturition, loosening of the teat sphincter occurs due to continuous synthesis and removal of milk. These

physiological changes alter immunity of the gland, thus making the buffaloes most vulnerable to new Intra mammary infections one week after calving.

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