Scanning Electron Microscopy and Phagocytic Activity of Blood and Milk Neutrophils Isolated from Early Lactating Buffaloes

Article · July 2012

CITATIONS
2

READS
219

10 authors, including:

Dilip kumar Swain
Uttar Pradesh Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya …
100 PUBLICATIONS **343** CITATIONS
SEE PROFILE

Arpita Mohapatra
Central Sheep and Wool Research Institute
16 PUBLICATIONS **43** CITATIONS
SEE PROFILE

Mohsinkhan Pathan
Anand Agricultural University
38 PUBLICATIONS **161** CITATIONS
SEE PROFILE

Suman Kapila
National Dairy Research Institute
177 PUBLICATIONS **2,059** CITATIONS
SEE PROFILE

Research Article

Scanning Electron Microscopy and Phagocytic Activity of Blood and Milk Neutrophils Isolated from Early Lactating Buffaloes

Arpita Mohapatra, D.K. Swain, Sashipal, M.M.K. Pathan, Mandheer Kaur. Shakti Ranjan Panigrahy, Suman Kapila, Rajeev Kapila, Shiv Prasad, A.K. Mohanty and A.K. Dang*

National Dairy Research Institute, Karnal-132001 (Haryana)

Neutrophils are considered as the first line of cellular defence in the mammary gland. Neutrophils are more active in blood but once they cross the mammary barrier, their activity gets reduced significantly. This reduction in the neutrophilic activity makes the mammary gland more susceptible to infections. The decreased activities of the neutrophils are associated with altered structural and functional integrity of the neutrophils. No information is available regarding the alterations in surface architecture of the blood and milk neutrophils in early lactating buffaloes. The current study was designed in Murrah buffalo cows during early lactation to evaluate the structural alterations in both blood and milk neutrophils. Scanning electron microscopy was carried out to evaluate the surface dynamics of the neutrophils. Phagocytic activity was also estimated to correlate the changes in ultrastructure with activity of neutrophils. The study concluded that the surface of blood neutrophils were more ruffled as compared to that of milk neutrophils. Reduced ruffledness of the membrane of milk neutrophils indicated a decreased neutrophilic activity and hence the chances of increased infection of mammary gland during early lactation.

Keywords: SEM, blood, milk, neutrophils, ruffled surface, early lactation, phagocytosis

INTRODUCTION

Neutrophils are classified as poly-morphonuclear cells (PMN) which serve as the first line of cellular defense. The dynamic crosstalk between invading pathogens, endotheliocytes, epithelial cells and neutrophils serves an integral part of host innate immune defense mechanism (Paape *et al.* 2003). The physiological structure and function of neutrophils mediates the intrinsic protection to the host body. Milk neutrophils are the key players in providing immediate and sustained protection to mammary glandular cells from the invading pathogens. Milk neutrophils originate from the migrating blood neutrophils at the mammary epithelial interface (Burton *et al.* 2005). The process of neutrophilic migration into mammary gland is a complex signaling process which is initiated due to invaded microbes, localized tissue damage and chemokine mediated chemotaxis. Neutrophils cross the epithelial border to reach to mammary cells for carrying out phagocytosis (Pyorala, 2003).

Mammary neutrophils exhibit an advanced stage of development as compared to blood neutrophils which exhibit both matured and immature stages. Blood exhibits more number of band cells and immature neutrophils whereas milk does not exhibit immature stages of neutrophils. Recent studies have revealed that milk neutrophils show a lower life span as compared to that of blood neutrophils. Milk neutrophils also exhibit a fundamentally compromised phagocytosis and oxidative burst. Studies from our laboratory indicated a reduced in vitro phagocytic ability of mammary neutrophils as compared to blood neutrophils (Vishnoi *et al.*, 2007).

Currently work is being done to improve the immune activity of neutrophils as they are considered to be the first line of defence and prevent the establishment of pathogens in the

* *Corresponding Author: Dairy Cattle Physiology Division, National Dairy Research Institute, Karnal, Haryana-132 001, India, Tel: 0091-184-2259084; Fax: 0091-184-2250042; E mail: rajadang@rediffmail.com*

2012-079 Received:July 2012 ; Accepted:December 2012

mammary gland. Transmission electron microscopy studies have revealed that milk neutrophils contain less number of secondary granules which are associated with phagocytic ability of neutrophils (Paape *et al.*, 2003). This may be a factor for poor phagocytic activity of the milk neutrophils. In the current scenario, milk neutrophils are targeted to increase the physio-immune status to achieve maximum efficiency. Surface architecture of neutrophils is involved in both signaling as well as in the process of phagocytosis. Neutrophils exhibit a highly ruffled surface by exhibiting pseudopods on the surface. This increases the surface area of neutrophils and helps in augmenting phagocytosis by increasing the formation of phagosomes (Mehrzad *et al.*, 2004).

Scanning electron microscopy (SEM) serves as one of the best tool to study the cell surface architecture. The technique helps in the elucidation of key membrane features of the cells. Studies have been carried out by using SEM to observe the surface of both bovine and caprine neutrophils isolated from milk and blood (Tian *et al.*, 2005).

Calving and early lactation periods impose a higher degree of physiological stress on dairy animals (Erskine, 2001). Stress is also induced by high levels of cortisol which causes immune suppression as well as decreases mammary defense. That is why calving period and early lactation period are critical determinants of future immune response and protection of mammary gland. Due to compromised immune activity, neutrophil functions get altered. Although buffaloes are less prone to mastitis due to their tight sphincter and larger teat length but the exploitation of these animals for higher milk production using extensive management practices have made them more susceptible to mastitis (Dang *et al.*, 2007). So far there is no report about the architectural alterations in blood and milk neutrophils of buffaloes. Over viewing these, the present study was designed to evaluate the membrane architecture of buffalo neutrophils isolated from buffalo blood and milk during early lactation periods.

MATERIALS AND METHODS

The present study was performed in the cattle yard of NDRI, Karnal. Six clinically healthy Murrah buffalo cows which were free from mastitis were taken. Blood and milk samples were collected after exactly 10 days of calving at every alternate days and continued to upto 30 days.

Blood and Milk Sampling

Milk and blood samples were collected as per the protocols approved by the animal ethics committee of the Institute. Teats were disinfected with 70% of ethyl alcohol prior to collection of milk. 50 ml of milk was collected in sterile plastic centrifuge tubes. The tubes were collected in ice box and immediately transferred to the laboratory for further processing and evaluation. Autologous blood samples of 10 ml were taken after sampling of milk by venipuncture from the external jugular vein using heparin as an anticoagulant.

Isolation of Blood and Milk Neutrophils

All materials and reagents used for the isolation of blood and milk polymorphonuclear leukocytes (PMN) were sterile. Isolation of PMN from peripheral blood was performed using hypotonic lysis of erythrocytes as described by Mehrzad *et al*. (2002 and 2004). Briefly, 10 ml of Ethylenediaminetetraacetic acid mixed blood was poured into the Falcon tubes and centrifuged (1000 X g, 15 min., 4°C); the plasma layer, buffy coat, and top layer of the blood-packed cells were discarded. About 2.5 ml of the blood-packed cell was lysed by adding 5 ml of double distilled water and gently mixed for 45 sec. using a magnetic stirrer. After restoration of the isotonicity the suspension was again centrifuged (1000 X g, 10 min., and 4°C). For the second lysis procedure, after resuspending of the pellets in 2.5 ml of Dulbecco's PBS, 2.5 ml of 2.7% NaCl was added, gently mixed for 60 sec., and centrifuged (1000 X g, 5 min., 4°C). The remaining cell pellet was washed 3 times in PBS (300 X g, 10 min., 4°C) and the final cell pellet was resuspended in RPMI media for further analysis.

Isolation of PMN from milk was performed as per method described by Mehrzad et al. (2002) in milk samples. Briefly the milk was filtered separately through a nylon filter (40 µm pore size) and diluted to 60% with cold Dulbecco's PBS (volume/volume). Isolation of PMN was performed using 3 centrifugations steps. 10 ml of milk was poured into a centrifuge tube and

centrifuged (600 X g, 15 min., 4°C). The fat was removed and the remaining cell pellet was washed twice in cold Dulbecco's PBS (300 X g, 10 min., 4°C and 200 X g, 15 min., 4°C). The isolation procedure of PMN from blood yielded >98% of granulocytes (PMN + eosinophils) with predominantly PMN (>85%) as determined by counting the cells in smear stained with Leishman's stain. The viability was determined using Trypan Blue exclusion test.

The cell suspension of neutrophils was adjusted to 5 x 10_6 live cells/ml by the culture media (RPMI 1640) containing 10% FCS. 200 l of the diluted cell suspension per well in triplicate was placed in a 96 well flat bottomed tissue culture plate. The cells were allowed to proliferate with Zymosan (650 µg/ml) and NBT (250 µg/ml) concentrations. All cultures were allowed to incubate at 37°C in a humidified CO_2 incubator (95% air and 5% CO_2) for 2h. Amount of zymosan phagocytosed was used as an indicator of phagocytic activity. Nitroblue tetrazolium (NBT) assay was used to determine the production of superoxide anion (O2-) in the neutrophils. NBT is yellow in color, but is changed to blue formazan after phagocytosis which can be measured spectrophotometrically (Dang *et al.,* 2010 and 2012).

Processing of Samples for Scanning Electron Microscopy

Investigation of ultra structural changes of both blood and milk neutrophils was carried out by employing SEM as previously described by Tian *et al.,* (2005) with minor modifications. The same protocol was used for both blood and milk neutrophils. Briefly, the freshly isolated cells were prepared for microscopy in three steps like fixation, dehydration and coating. Freshly isolated neutrophils were added to 0.7 ml PBS and 0.3 ml of fixative solution (2.5% Glutaraldehyde). The mixture was thoroughly mixed and centrifuged at 1500 RPM for 10 minutes. After centrifugation, the supernatant was removed and to the pellet fresh 0.5 ml of fixative was added. The mixture was kept at 4°C for 2 hrs. This was followed by removal of fixative by centrifuging at 1500 RPM for 10 minutes. 1 ml PBS was added after the removal of supernatant.

The pelleted cells were kept undisturbed for next step of dehydration by graded acetone. 0.5 ml of 30% of acetone was added to settled cells. Pellet was properly mixed with acetone and suspended in eppendorf tube for 10-20 minutes. The mixture was centrifuged and the supernatant was removed. Similar procedure was carried out with 50%, 70%, 90% and 100% acetone dehydrate the cells. Finally the cells were kept in 0.5 ml of 100% acetone and vortexing was carried out. Carbon paper was kept on stubs and cover slips were kept over it. About 10 µl of Hexamethyl-derilazane (HMDL) was added. The sample was diluted with 1.5 ml of 100% acetone and 20 µl of the sample was taken for drying. Pallidium coating was carried out for 1½ hrs. The coated samples were examined under scanning electron microscope (Zeiss Evomaio) at 20KV/EHT and 10Pa between 1500X to 2000X after 30nm palladium coating.

RESULTS AND DISCUSSION

Morpho-architectural assessment of blood and milk neutrophils was carried out by SEM. The sampling was carried out from buffaloes during early lactation (10 to 30 days after calving). The scanned images obtained from blood neutrophils have been presented in Plate A1 and A2 and that of the milk neutrophils have been presented in Plate B1 and B2. Neutrophils from both the samples exhibit almost a similar size ranging between 10 to 15 µm in diameter. SEM revealed both the neutrophils are round in shape. Blood neutrophils exhibited ruffled surface as compared to the neutrophils derived from milk. Milk neutrophils appeared smoother, but on higher magnification, milk neutrophils exhibited more ruffled surface as compared to blood neutrophils. Tian *et al.*, (2005) reported that milk neutrophils were having more irregular surface as compared to the blood neutrophils at peak lactation as peak lactation exhibits more immune stress as compared to early lactation. This may be a probable cause for getting contrasting results in our study.

We have collected milk and blood neutrophils during early lactation period just after calving. During this period, plasma cortisol levels remain at a high level which is the major cause behind immune suppression (Erskine, 2001). This may be the reason that the animals are highly susceptible for a number of infections during early lactation. This is also relevant from our current study of neutrophil structure by SEM.

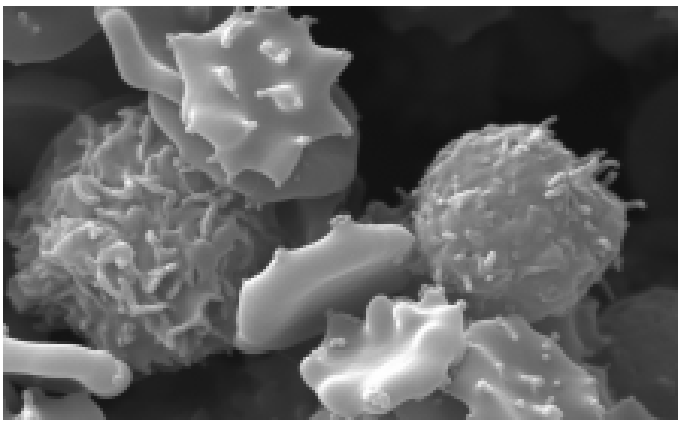

PLATE A1: SEM of blood neutrophils exhibiting ruffled Surface (2000 X)

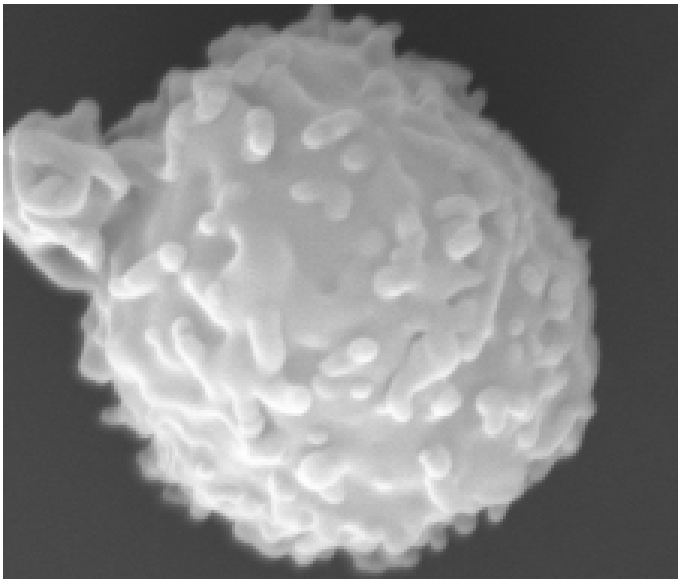

PLATE A2: SEM of blood neutrophils exhibiting ruffled Surface (3500 X)

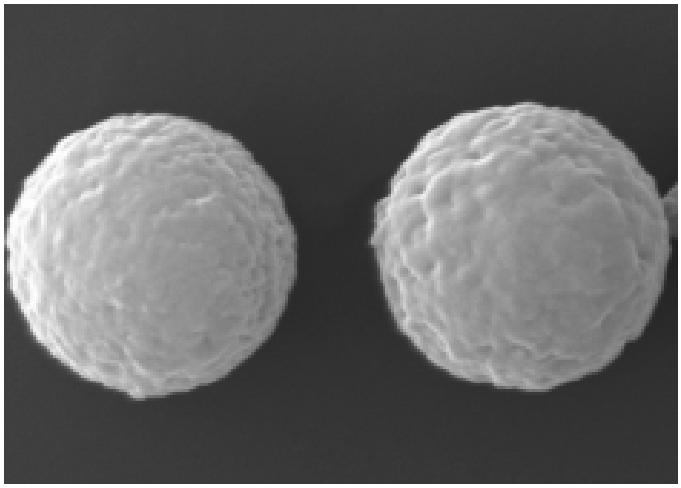

PLATE B1: SEM of milk neutrophils exhibiting less ruffled Surface (2500 X)

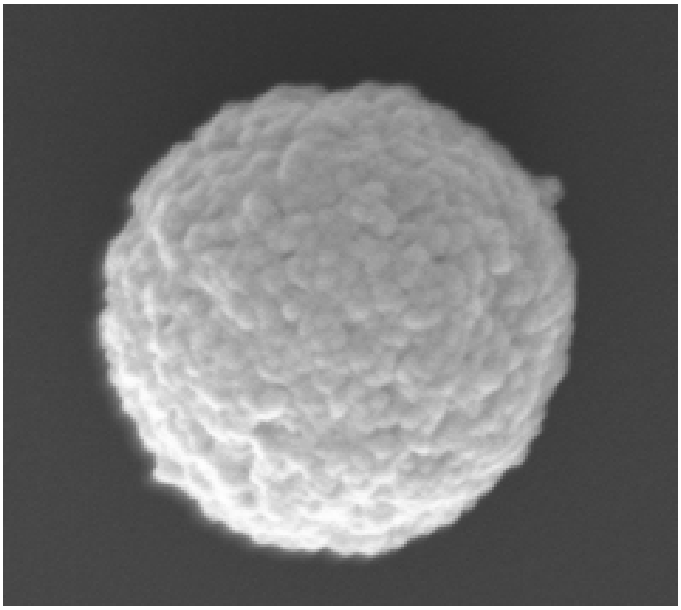

PLATE B2: SEM of milk neutrophils exhibiting less ruffled Surface in High magnification (3500 X)

Smooth surface of milk neutrophils is the indication of decreased immune status of the animal which may be due to the peripartum period as well as the stress due to the parturition.

Paape *et al*. (2003) extensively reviewed bovine neutrophil ultrastructure during peak lactation and reported highly irregular surface of milk neutrophils as compared to blood neutrophils. In contrast to this, our study revealed a smooth neutrophil surface. This may be partly due to different stage of lactation; secondly we have taken buffalo as our species of study and thirdly we adopted a modified and simpler method for SEM for both milk and blood neutrophils. However, further studies are required to in a large number of animal samples in different stages of lactation so as to find out why there is alteration in both structural and functional organization of milk and blood neutrophils and how it affects the functions of neutrophils.

Further studies may also be carried out in different phases of lactation cycle to elucidate the key changes in neutrophil structure. To validate our SEM findings, we carried out in vitro phagocytic assay of both blood and milk neutrophils (Fig A). Milk neutrophils during early

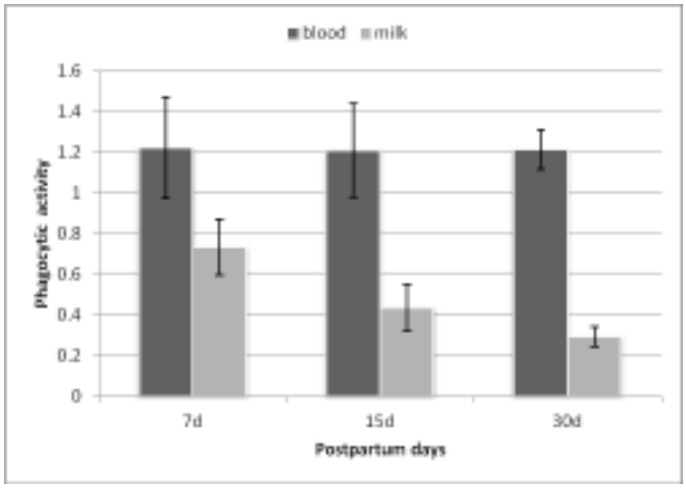

Fig 1. Phagocytic activity of blood and milk neutrophils during early lactation in Buffaloes

lactation exhibited a decreased phagocytic activity as compared to the blood neutrophils. We marked a remarkable and significant reduction in phagocytic activities of milk neutrophils from 10 to 30 after calving. This was correlated with our SEM finding of blood and milk neutrophils. Diminished phagocytic activity of milk neutrophils revealed a smooth membrane of milk neutrophils where as blood neutrophils exhibited more ruffled membrane and enhanced phagocytic activity.

This is the first study during early lactation where neutrophil ultra structure has been studied in buffaloes. Our results could not be compared as no literature is available on SEM of neutrophils during early lactation in dairy animals. That is why the study is unique to report about the neutrophil activities during early lactation and why an animal becomes more prone to infection during this period. Further studies are required to study neutrophil activities and structure throughout early lactation so as to improve the immune competence of milk neutrophils and to develop a safe guard to the mammary infections.

ACKNOWLEDGMENTS

The authors are thankful to Dr. V.V. Ramamurthy, Dept of Entomology, IARI, New Delhi for helping all facilities to carry out SEM of blood and milk neutrophils. We are highly thankful to the Department of Biotechnology, Ministry of Science and Technology, India for providing financial support to carry out this study.

REFERENCES

Burton, J. L., Madsen, S.A., Chang, L.C., Weber, P.S.D., Buckham, K.R., Dorp, R.V., Hickey, M.C. and Bernadette, E. 2005. Gene expression signatures in neutrophils exposed to glucocorticoids: A new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows. *Vet. Immun. Immunopath.* **105**:197-219.

Dang, A.K., Kapila, S., Tomar, P. and Singh, C. 2007. Immunity of the buffalo mammary gland during different physiological stages. *Asian Austr. J. Anim. Sci.* **20**(8):1174-1181.

Dang AK, Mukherjee J, Kapila S, Mohanty AK, Kapila R and Prasad S.2010. In vitro phagocytic activity of milk neutrophils during lactation cycle in Murrah buffaloes of different parity. *J. Anim. Phy. Anim. Nutr.* 706-711.

Dang AK, Prasad S, De K, Mukherjee J, Sandeep IVR, Mutoni G, Pathan MM, Jamwal M, Kapila S, Kapila R, Kaur H, Dixit S, Mohanty AK and Prakash BS. 2012. Effect of supplementation of Vitamin E, copper and zinc on the in vitro phagocytic activity and lymphocyte proliferation index of peripartum Sahiwal(Bos indicus) cows. *J. Anim. Phy. Anim. Nutr.* (Article first published online: 31 JAN 2012, DOI: 10.1111/j.1439-0396.2011.01272.x).

Erskine RJ. 2001. Enhancing immunity during the dry period : pitfalls and opportunities. National Mastitis Council Annual Meeting Proceedings. pg 95-106.

Mehrzad J, Duchateau L, Pyorola S and Burvenich C. 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. *J. Dairy Sci.* **85**:3268-3276.

Mehrzad J, Duchateau L and Burvenich C. 2004. Viability of Milk Neutrophils and Severity of Bovine Coliform Mastitis. *J.Dairy Sci.* **87**:4150-4162.

Paape MJ, Bannerman DD, Zhao X and Lee JW.2003. The bovine neutrophil: Structure and function in blood and milk. *Vet. Res.* **34**:597-627.

Pyorala S. 2003. Indicators of inflammation in the diagnosis of mastitis. *Vet. Res.* **34**:565-578.

Tian SZ, Chang CJ, Chiang CC, Peh HC, Huang MC, Lee JW and Zhao X. 2005. Comparison of morphology, viability and function between blood and milk neutrophils from peak lactating goats. *Can. J. Vet. Res.* **69**:39-45.

Vishnoi, P.C., Dang, A.K. and Kapila, S. 2007. *In vitro* phagocytic activity of neutrophils in blood, normal and infected milk of Murrah buffaloes. *Buffalo J.* **1**:51-59.