

ISOLATION OF EXFOLIATED SOMATIC CELLS FROM BUFFALO MILK

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ABSTRACT

A technique for isolation of leucocytes (neutrophils, macrophages and lymphocytes) and epithelial cells from buffalo milk was successfully carried out. Higher fat and total solids in buffalo interfere with the isolation of milk cells, but an increase in the duration and speed of centrifugation helps in the successful isolation of these cells. Isolation of epithelial and milk leucocytes from milk is a non-invasive, easily repeatable technique which allows frequent sampling and can be used to study the immune and synthetic activity of exfoliated milk cells *in vitro*.

Keywords: buffalo, milk, neutrophils, macrophages, lymphocytes, epithelial cells

INTRODUCTION

Milk somatic cells are simply animal body cells present at low levels in normal milk. These cells include both the leukocytes and the epithelial cells which are sloughed off during the normal process of milking throughout the lactation cycle (Harmon, 1994). The epithelial cells are capable of synthesizing milk, whereas, the white blood cells serve as a defense mechanism to fight disease (infection), and assist in repairing damaged tissue. Various leucocytes and epithelial cells can

be isolated and studied for their activity from the milk cell pellet after centrifugation at low speed (Boutinaud and Jammes, 2002). After isolation, milk somatic cells can also be used for dynamic studies of gene expression in the mammary gland. Isolation, culture and determination of activity of each milk leucocyte can also help in assessing mammary gland immunity and may be used as a tool for genetic selection of high-producing animals. Successful isolation and culture of neutrophils from colostrum and milk from buffaloes has been developed (Dang *et al.*, 2010). But there is no report on the isolation of milk lymphocytes, macrophages and epithelial cells from milk of buffaloes, which are the major contributors of milk production in South Asia. Also, buffalo milk is different from that of cows as it has more fat and total solids, which prevent proper isolation and culture of milk cells. Therefore, the main objective of the present study was to isolate and culture exfoliated cells from the milk of buffaloes.

MATERIALS AND METHODS

Milk samples were collected from healthy Murrah buffaloes having normal somatic cell counts (Dang *et al.*, 2010). Udder and teats of selected buffaloes were washed and cleaned with 70% absolute alcohol. Milk was collected in

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sterilized containers and processed within 2 h of milk sampling.

Isolation of polymorphonuclear neutrophils from milk

For isolation of polymorphonuclear neutrophils, fresh milk was filtered 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 four centrifugation steps as buffalo milk is higher in milk fat. Ten milliliters of milk was poured into a centrifuge tube and centrifuged (800X g, 20 minutes, 4°C). The fat was removed with supernatant. The remaining cell pellet was washed thrice in cold Dulbecco's PBS (500X g, 10 minutes, 4°C, 300X g, 20 minutes, 4°C and finally at 300X g, 20 minutes, 4°C). The final pellet was resuspended in Dulbecco's PBS containing 0.5 mg/ml gelatin. Addition of gelatin maintains the integrity of the neutrophils.

Isolation of lymphocytes from milk

Isolation of lymphocytes from milk was done by density gradient centrifugations. Milk was centrifuged (600X g, 20 minutes, 4°C) and the fat layer accumulated on top was removed along with the skim milk. The cell pellet obtained at the bottom was dissolved in PBS. This was layered over the Histopaque -1077 and was centrifuged at 600X g for 20 minutes at 4°C. The fat layer on top was removed and the whole white layer of lymphocytes above the Histopaque was collected in which lymphocytes comprised the major portion of DLC (69-71%), whereas, monocytes were found to range between 1-3%. It was washed once with PBS and finally once with media.

Isolation of macrophages from milk

For isolating macrophages, milk was placed

into sterile 50-ml siliconized tubes and diluted 1:4 with PBS and then centrifuged at 4°C and 500X g for 15 minutes. The supernatant milk was recentrifuged to ensure maximum yield of cells per sample, and the pellets were combined. Cells were layered onto Ficoll-Hypaque and centrifuged at 500 X g for 30 minutes. The mononuclear cell layer was washed twice with PBS and suspended in Dulbecco's modified Eagle's medium containing penicillin and streptomycin. Cells were allowed to adhere to tissue culture dishes for 1 h at 37°C. Adherent cells were washed vigorously twice with HBSS. After the adherence, cells were assayed immediately or incubated overnight in medium. Adherent cells were found to be 90 to 95% macrophages by May-Grünwald stain.

Isolation and culture of epithelial cells from milk

Briefly, milk samples were collected from buffaloes and centrifuged at 3500 rpm, 20°C for 15 minutes. The supernatant was discarded and the remaining milk cell pellet was resuspended in buffer. The milk was again centrifuged for 5 minutes at 3000 rpm. The supernatant was discarded and the remaining milk cell pellet was again resuspended in buffer. The cell pellet was resuspended in Dulbecco's Modified Eagle's Medium and filtered through a nylon cell strainer. After another centrifugation at 3000 rpm, the remaining cell pellet was resuspended in 10 ml pre-heated medium (37°C). The medium with the cells were kept in sterile tissue culture bottles. The medium was changed twice per week. The cells appear to be inert for the first two days of culture, after which both small and large cells adhered to plastic dishes, making it possible to distinguish them from lymphocytes and granulocytes, which remained in suspension.

RESULTS AND DISCUSSION

The present study was conducted for the first time to isolate milk macrophages, neutrophils, lymphocytes and epithelial cells from milk of buffaloes. The results of the smears of milk neutrophils, lymphocytes and macrophages have been presented in Figures 1, 2 and 3, respectively. Whereas, the results obtained after culturing of milk neutrophils, lymphocytes, macrophages and epithelial cells have been presented in Figures 4, 5, 6 and 7, respectively. All the culture photographs were taken at 1000 X magnification under oil immersion. The viability of all milk leukocytes was determined using Trypan Blue, after counting the cells using a hemocytometer. The isolation procedure of PMN from milk yielded >80% of granulocytes (PMN + eosinophils) with predominantly PMN (>80%) as determined by counting the cells in smears stained with Leishman's stain. The viability of milk lymphocytes in different experiments was found to range between 95-98% within 6 h of processing and declined gradually afterwards. The viability of milk macrophages was found to be around 91-93% after washing and declined afterwards. Very few live epithelial cells were present in buffalo milk, but all these cells were able to proliferate and terminally differentiate into small colonies. The presence of mammary epithelial cells in the culture was confirmed further by immunostaining. On day 30, three different cell colonies of epithelial cells were seen; tightly joined elongated cells, tightly joined cuboidal cell colonies and contiguous cells were observed.

Our results indicate that as in cows (Mukherjee and Dang, 2011) various cells can also be isolated from buffalo milk after increasing the duration and number of centrifugation steps. Further, by isolating milk phagocytes, i.e. both

macrophages and neutrophils, and challenging them with bacteria, their phagocytic activity can be estimated as is done for blood neutrophils (Dang *et al.*, 2007). Lymphocyte proliferation assay can be studied from the isolated milk lymphocytes by stimulating them with various mitogens as done for blood lymphocytes. Also live epithelial cells could also be harvested from buffalo milk as reported in cow's milk (Buehring, 1990) and used for determining their synthetic and immune activity.

Of all the leucocytes, neutrophils are the first ones to respond (Jain, 1986) and migrate from blood circulation to an inflamed area where they phagocytose and kill bacteria. As neutrophils travel from the blood to the mammary gland their activity is reduced which increases the chances of intra mammary infections (Paape *et al.*, 2002). This is because milk neutrophils have reduced abilities to produce reactive oxygen species, when compared to blood (Goldbery *et al.*, 1995; Dosogne *et al.*, 2001). Also diapedesis of blood PMN through the blood-milk barrier causes a reduction of the phagocytic and oxidative burst activity was also demonstrated using an *in vitro* cell culture model (Smits *et al.*, 1999). Therefore, for selection of high-producing buffaloes we can isolate individual neutrophils, macrophages and lymphocytes and estimate their immune activity. Only buffaloes having higher production potential along with improved immune function should be selected. Selection done on this basis may reduce the incidence of subclinical or clinical mastitis in high-producing buffaloes.

Above all, the above technique of isolation of milk somatic cells is a non-invasive technique. In the future, this technique not only will help us in the better understanding of the immune regulation in the mammary gland but may also lead to improved treatments and vaccine development for our high-producing buffaloes.

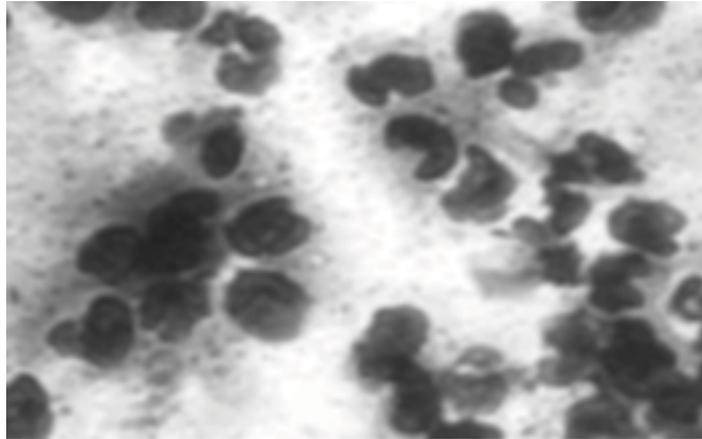


Figure 1. Milk neutrophils (40X).



Figure 2. Milk lymphocyte (40X).

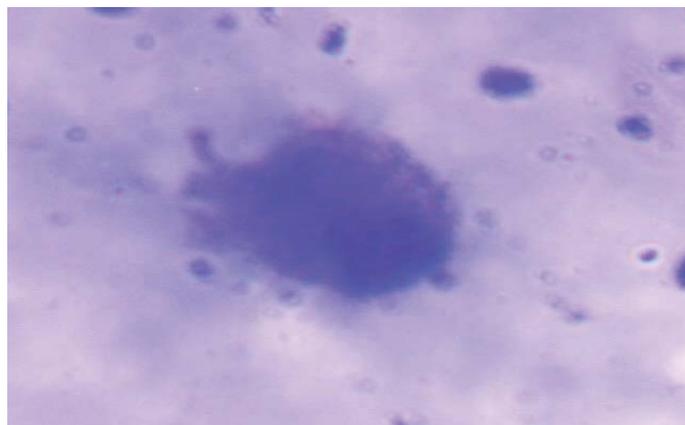


Figure 3. Milk macrophage (100X).

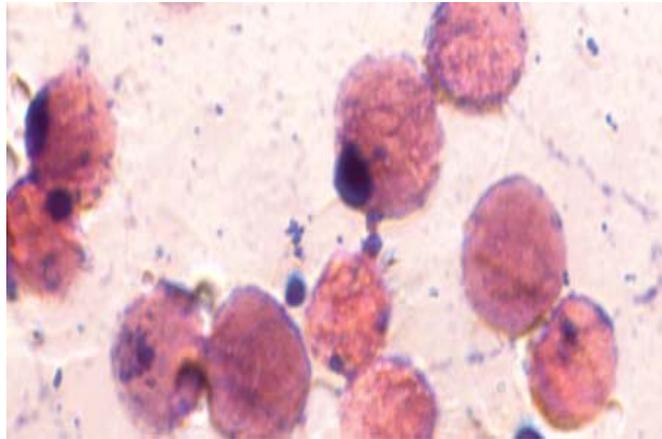


Figure 4. Culture of milk neutrophils (6 h) (1000X).

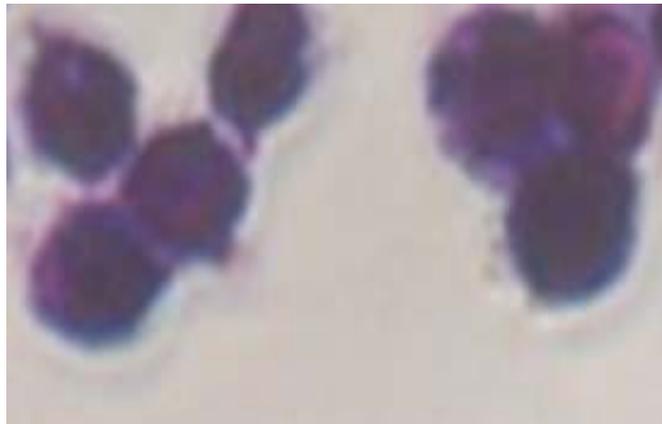


Figure 5. Culture of milk lymphocytes (12 h) (1000X).

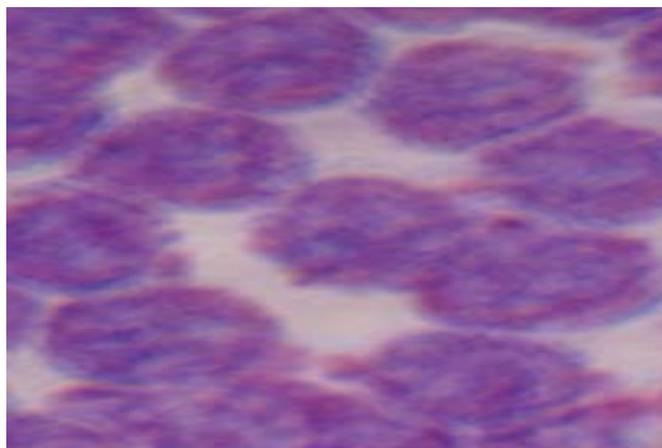


Figure 6. Culture of milk macrophages (24 h) (1000X).

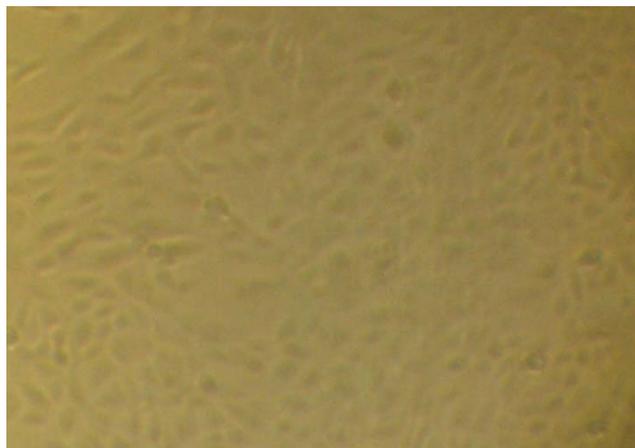


Figure 7. Culture of milk epithelial cells (45 days) (1000X).

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