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A study of changes in colostrum and transition milk immune cells along with growth factors in dairy cows

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Abstract
First lacteal secretion is known as colostrum or liquid gold and is the single most important factor in determining health and survival of the neonatal calf. Colostrum contains high concentration of nutrients and biologically active components such as carbohydrates, growth factors, enzyme inhibitors, nucleotides, cytokines, fats, minerals and vitamins, as well as immune-competent cells and soluble proteins that provide immunity to the infant and influence immune system maturation. The sole objective of colostrum is to fulfil the nutritional, immunological and growth demand of new born calf. Somatic cell count (SCC) in colostrum was very high in first few hrs after its number reduced gradually. Somatic cells and differential leukocyte count (DLC) were estimated by making a smear on microscopic slide. Highest level of total SCC was reported in colostrum of day 0 (day of calving) then it decreased significantly on subsequent days, and lowest level was reported on the day 6. Colostrum contains highest percentage of macrophage fallowed by lymphocytes and neutrophils. In transition milk lymphocyte percentage increased significantly and reached highest level on the day 6, whereas number of macrophages was decreased significantly and reached lowest level on the day 6. However, no significant difference was reported in case of neutrophils of colostrum and transition milk. Viability of somatic cells in colostrum of day 0 was significantly (P<0.05) low as compare to other days of lactation. The level of IGF1 was significantly (P<0.05) changes from day 0 to days 6, whereas there was no significant difference between days 5 and 6. The current study thus provide the basic information related to the concentration of different cells, viability and growth factors in colostrum and transition.

Keywords: colostrum, somatic cell count, transition milk, growth factors

Introduction
The early milking from dairy cows, taken from first 3 days and 3-6 days post-partum is consider as colostrum and transition milk (produce after colostrum and before whole milk) respectively. From ancient time in India, the physicians of Ayurvedic prescribed colostrum and present time colostrum stills very often used as therapeutic purpose by many families (Rocha, 2016) [34]. Colostrum is known as white gold due to its composition and important for infant (Dang *et al.*, 2009) [5]. Feeding colostrum within few hours provide passive immunity and other bioactive substance which is highly essential for immediate and future prospective. Colostrum is rich in nutrients and non-nutrient biologically active components, including carbohydrates, growth factors, enzymes, enzyme inhibitors, nucleotides and nucleosides, cytokines, fats, minerals, vitamins along with immune-competent cells and soluble proteins that provide immunity to the infant and affect the maturation of the infant's immune system by McGrath *et al.*, 2016 [21]; Hammon *et al.*, 2020 [13]. The sole objective of colostrum is to fulfil the nutritional, Immunological and growth demand of new born calf. It is of more importance in a new born calf of ruminant because during foetal condition immunoglobulin are not transferred from the dam to fetus because ruminant have epitheliochorial/syndesmochroial (syncytium junction) type placenta that prevent transfer of passive immunity from dam to fetus (Borghesi *et al.*, 2014) [3]. Hence colostrum become very important source for delivery of immunogenic component to the calf survival after parturition.
SCC in colostrum is very high in first few hrs after latter its number reduces gradually. Jeong *et al.* (2009) [14] reported that the SCC of colostrum was highest initially and decreased gradually over the first 132 h after parturition. In case of subclinical and clinical mastitis count of SCC increase (Swain *et al.*, 2014) [38] so it's no used as a direct indicator of milk quality and non-invasive method of udder health of the animal, after first weeks of parturition, presence of higher no of cells is a physiological not a pathological condition.

Many previous studies proved, the presence of higher no of colostral SCC provide passive immunity by crossing the neonatal intestinal barrier, it has since been described in many species including mice (Stieler *et al.*, 2012) [37], cattle (Liebler-Tenorio *et al.*, 2002) [20].

Bovine colostrum contains around 10^6 leukocytes/mL, which contribute about 98% of total somatic cell count. Differential leukocyte counts (DLC) can be used for a more comprehensive study of the udder health status. The population of leukocytes in colostrum consists of granulocytes and mononuclear cells, including neutrophils, macrophages and lymphocytes. Neutrophils and macrophage provide defence to mammary gland against invading pathogen by phagocytosis. In cattle lymphocytes proportion is highest in milk which account 62% and in colostrum 22-25% (Yang *et al.*, 1997) [39]. In colostrum macrophage is dominant cell and its proportion ranges 35-79% and in milk it is about 21% (Ostensson *et al*., 1998) [24]. Polymorphonuclear cells account for 3% to 26% and epithelial cells account for 2% to 15% of the total cell population (Paape and Tucker, 1996 [26]; Yang *et al.*, 1997 [39]; Kelly., 2000 [15]). Although that value varies depending on the age, breed, health and immune status of individual cows. Change in leukocyte cells population from colostrum to transition milk at consequent days is unclear with the advancement of lactation is unknown. There has been lack of information on the changes in individual populations of leucocyte types in colostrum and transition milk (3-7 days) of dairy cattle.

Viability is another important factor which indicate the health status of mammary gland. Number of live and dead cells of colostrum and milk vary with frequency of milking, parity, immune status and pathological condition of animal. In many previous studies has been explore the ratio of live dead cell count in subclinical and clinical mastitis condition and reported change in leukocyte papulation and viability and phagocytic activity of cells by Swain *et al.*, 2014 [38]; Alhussien *et al*., 2015 [1]. During early lactation cattle passes through immune suppression which leads to increase chance of mastitis and other metabolic diseases. There has been lack of information on the fraction of live and dead cells populations of in colostrum and transition milk (3-7 days) of dairy cattle. Early detection of prevalence is of mastitis can be beneficial for dairy worker but unfortunately there is no data available about how SCC live and dead cells papulation/ratio changes in colostrum and transition milk which can be used as reference for approximation occurrence of mastitis and milk quality.

Colostrum is enriched with various bioactive compound, IGF1 is one of the important growth factors which imparts important biological function like angiogenesis, cell proliferation and GIT development in calves after birth. Its concentration is highest in first few hrs of milking and then decreases very sharply. At the time of first milking, it ranges 150-2000ng/ml and in mature milk it is 25-35ng/ml (Gauthier *et al*., 2006) [10].

Materials and Methods

Thirty-five (3 ± 1 parity) advanced pregnant clinically healthy and without previous history of mastitis Karan-Fries (KF) (cross of Tharparkar and Holstein-Friesian cows) were selected from Livestock Research Centre of National Dairy Research Institute, Karnal, Haryana. Cows in their late gestation at 45 days before the expected date of calving were selected. Pregnant cows with average body weight (510 ± 12.58 kg), BCS (3.12 ± 0.09), parity (2.8 ± 057) and colostrum yield, 8.22 ± 0.73kg. (Receiving a basal diet).

Table 1: Composition of the concentrate mixture (Cows)

Ingredients	Parts (%)
Maize (cracked)	33
Mustard cake (oiled)	12
GNC (oiled)	21
Wheat bran	20
Deoiled rice bran	11
Mineral mixture	2
Common salt	1

Sample collection

Sample was collected from time of parturition till 6 days of lactation at every 24 hrs. Before milking, teat ends were scrubbed with 75% ethanol and the initial two squirts of colostrum/milk were discarded. Individual colostrum samples pooled from all four quarters of the animal were collected separately and aseptically in clean milk bottles. The samples were brought to the laboratory immediately after collection for analysis.

Colostrum Somatic cell counts

SCC of colostrum and transition milk samples were measured by a somatic cell counter (Milkotronic Ltd., Stara Zagora, Bulgaria) and also cross checked by making smear. The fresh colostrum and transition milk collected in 50 ml of Falcon's tube kept at room temperature 15-25˚C and gently mixed well, and100 µl was taken in micro-tube with Sofia Green lyophilized dye and stirred the sample using Mini Vortex. Careful stirring was done for 9-10 times for every 1-2 seconds. The sample was incubated for 90 second for interaction of milk with dye. The stirring procedure was repeated carefully for 3-4 times for every 1-2 seconds using Mini-Vortex. 8 µl sample was taken through pipette in the microfluidic camera of the lactochip x4 (for 4 samples) or 14 µl sample was taken in the microfluidic camera of the lactochip x2 (for 2 samples). The sample lactochip x4/lactochip x2 were loaded in the cartridge of the lactoscan SCC. Using the software, the SCC started analysis in each boxes. After analysis, the lactochips were discarded. For microscopic estimating of SCC, the slides were prepared within 1 h of samples collection. 5 µl of colostrum sample taken on slide and smeared in 1x1 cm^2 area, then allowed for air dry. Air dried slide kept in 45 ^{0}C heated xylene for 30 minutes to remove fat globule. After half an hour, the slide was stain with methylene blue for 5 min, after washed with water, allowed to dry and observed under 45X to count individual cells (Dang *et al.* 2008) [6].

DLC (Differential cell counts) count

Differential cell counting was carried out microscopically by examine slide in 100X. the major immune cells secreted in the colostrum like lymphocytes, neutrophils and macrophages were count (Dang *et al*., 2009) [5]. Another parallel procedure was also carried out to count DLC of colostrum and transition milk. Due to higher viscosity isolation of colostrum somatic cell is difficult, so colostrum sample is diluted in 3:5 in DPBS. Milk samples were then centrifuged (Eppendorf centrifuge 5810 R) at 500 x g at 4 ^{0}C for 15 min (Li., 2015) [19]. Cream layers and supernatants were discarded and pellets were washed twice in DPBS+1% FBS by centrifugation at 400 x g at 4 °C for 10 min. Cell pellets were finally

resuspended in DPBS+1% FBS for DLC and viability count. For DLC analysis on the microscope slide, resuspended cells were spread over an area of 2x2 cm^2 and air-dried slide stain with pure May-Grunwald (2 min) and Giemsa solution (45 second). Evaluation of the slides followed using light microscopy and oil immersion (100-fold magnification). One-hundred cells of each slide were counted meander-shaped and differentiated into lymphocytes, macrophages and PMN. Cell identification carried out according to standard methods (Lee *et al.*, 1980) [17]. Lymphocytes were identified based on their circular form (5–10 µm) and the typical shape of the nucleus that almost fills the cell leaving a very thin rim of cytoplasm. Cells of 8-30µm in size containing a little nucleus and pale staining were considered as macrophages. The group of PMN was characterized as cells of 10–14 µm in size and segmented nuclei. They were intensely coloured and contained granular in the cytoplasm.

Viability

Viability of the colostrum somatic cell was evaluated by trypan blue (Sigma Chemical Co) staining. 50 µl aliquot of resuspended cells of the homogenous suspension was mixed with an equal amount of 0.4% trypan blue solution (w/v). 10 µl of the above mixture Sample was loaded in Neubauer chamber and examine under 50x in microscope and count the cell in all tertiary/secondary square. Dead cells take pink stain while live cells are without any stain.

% of viable cell count = total live cells (unstained)/ total cells (viable + dead cells) X 100.

Growth factor IGF1

The samples were collected from time of parturition till 6 days of lactation at every 24 hrs and brought to the laboratory immediately after collection for separation of whey. A 40 ml sample was taken in falcon tube and centrifuge the sample at 3600 x g for 36 min at 4 ^{0}C after that fat layer was remove gently and take the skim colostrum layer without disturbing the pellet. A 20 ml of sample was taken in a 100ml glass beaker, heat in water bath 37 ^{0}C and then 0.5ml of 0.5% rennet solution (250 mg rennet in 50 ml distilled water) (Cat #: R5876-10G, Sigma) was added. After 20 min clotted sample was mixed by a glass rod and centrifuge 1500 x g for 5 min then filtered through Whatman no.42 filter paper and stored at -20 ^{0}C for further analysis. The IGF1 and IgG analysis of colostrum whey were measured by enzyme-linked immunosorbent assay ELISA (Cat #: SEA050Bo, cloud-clone corp).

Statistical analysis

Statistical analysis was performed as per the standard procedure. Statistical analyses were performed using the SPSS 17.0 statistical software package. The means were compared using Analysis of Variance and presented as mean ± standard error. The minimum significant range of confidence was evaluated at 0.05 level.

Results and Discussions

Pattern of Somatic cell count from colostrum to transition milk:

Somatic cells were estimated from direct smear on microscopic slide and by somatic cell counter. Highest level of total SCC in colostrum of 0 h were decreases significantly at all the day, there was no significant (P<0.05) difference between day 3, 4 and 5, at day 6, SCC were lowest. At the days 0, 1, 2, and 3 of colostrum sample SCC mean value were 21.32 x 10^5, 13.09 x 10^5, 9.18 x 10^5, 8.47 x 10^5 cells per ml and in transition milk of days 4, 5 and 6, SCC mean value were 6.65 x 10^5, 6.21 x 10^5 and 5.24 x 10^5. The changes of SCC from calving to transition milk have been presented in Fig 1.

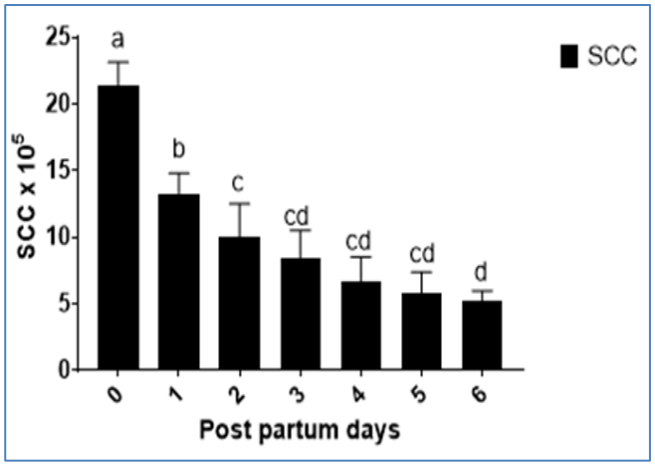

Fig 1: Changes of SCC from calving to transition milk

Somatic cell number was very high in zero hrs colostrum sample and its number was significantly (P<0.05) reduces with the advances of lactation. The somatic cell count of colostrum was ranges between 1x 10^6 to 3.5x10^6 (Ontsouka., 2003) [23] at the day of calving and its concentration were decreases linearly at 7 day its concentration reaches up to 5x10^5 which similar with the present study. Ontsouka *et al.* (2003) [23] reported that the mean SCC of colostrum on day 2 was 14.79 x 10^5 cells/mL which is similar with the present study 13.10±1.21x 10^5 cells/ml. Several previous studies reported SCC of colostrum is too much higher than mature milk (Andrew *et al.*, 2001) [2]. Presence of high somatic cell is not a pathological condition it is due to change in physiology of mammary gland, this increase number of cell is due to squeezing of cells through tight leaky junction between the mammary epithelial cells (Nguyen and Neville, 1998) [22]. Gradual reduction in the number of SCC from day of calving to transition milk may be due to closure of leaky junction. Bovine colostrum comprises approximately 10^6 leukocytes/ml although that amount and subtypes varies depending on the age, breed, health, and immune status of individual cows (Gonzalez *et al.*, 2017) [12]. Higher level of colostrum SCC and leukocyte may be a natural phenomenon because ingestion of colostrum containing maternal leukocytes are absorbed through the intestinal wall, enter the neonatal circulation and secondary lymphoid tissues, and play critical for stimulation of the immune system of new born calves (Reber *et al.*, 2005, 2006) [32, 33].

Pattern of differential leukocyte count from colostrum to transition milk:

DLC during different days of colostrum and transition milk of KF cows have been presented in fig 2. Macrophage was the most prominent leukocyte in colostrum and significantly varied (P<0.05) in different days its level went down in subsequent days with lowest 29.74% being found on the day 6 samples. At the day 0 and 1 shows highest macrophage cell count but there was no significant different between these two days. Compare with the 0 day there was significant (P<0.05)

difference at day 2, 3, 4, 5 and 6. and at day 6 its concentration reaches lowest level. Macrophage percentage at 0 day was 61.20% whare as at last day of transition milk it was 29.74%.

A significant (P<0.05) increased concentration of lymphocyte percentage was found in milk from 16.53% to 64.43% among colostrum samples of different days after calving.

Macrophage significantly varied (P<0.05) in colostrum samples of different days with highest 62% in 2 hrs colostrum samples. Then the level went down in subsequent days with lowest 24.90% being found in day 7 milk samples. A significant (P<0.05) increase was found in milk lymphocyte percentage from 16% to 51.43% among colostrum samples of different days after calving. The neutrophile percentage in first 3 days were 14-17% and from 4-6 days it was ranges 20-23% but there were no significant (fig. 2) difference from days 0 to 6.

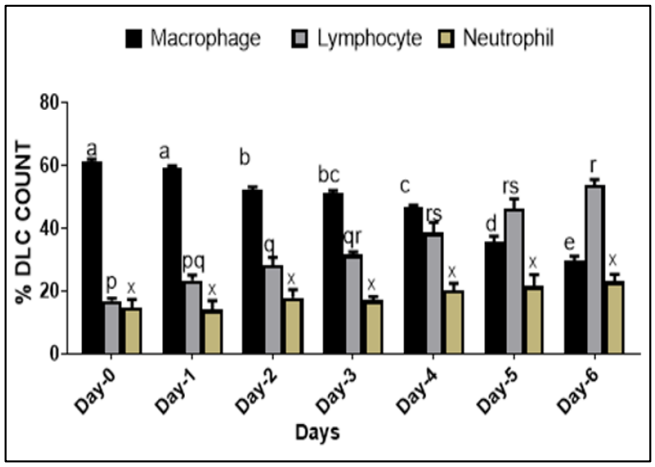

Fig 2: Pattern of differential leukocyte count from colostrum to transition milk

In healthy cow macrophages were most prominent leukocyte in colostrum fallowed by lymphocyte and then neutrophils up to first four days which is similar with Dang *et al*, (2009) [5] studies in buffalo. Similarly, many previous studies reported macrophage ranges from 40-50% in dairy cow colostrum sample (Liebler-Tenorio *et al*., 2002 [20]; Reber *et al*., 2005 [32]; Chase *et al*, (2008) [4]. Similarly, others like, Ellis *et al*., (1996) [9] and Ostensions *et al*., (1996) [25] reported its counts ranges 50-90% and 32-79% respectively. High level of macrophage in colostrum may be due to removal of debris that accumulate during dry period in mammary gland by phagocytosis, So migration of macrophage is more from peripheral circulation to mammary gland. Lymphocyte is second most dominant leukocyte in colostrum sample which mean was ranges 16-34% in colostrum sample, and in 4th to 6th days transition milk its were reaches 38 to 53%, which is concurrent with Liebler-Tenorio *et al*., 2002 [20]; Reber *et al*., 2005 [33]; Park *et al*., 1992 [30]; Schwarz *et al*, 2011 [35]. The present study is consistent with the earlier findings of Yang *et al*., (1997) [39] in which they reported 16% and 62% lymphocyte in colostrum and mature milk respectively, whereas Kelly *et al*., (2000) [15] reported macrophage, lymphocyte and neutrophils contribute 60%, 28% and 5-12% in mature milk, that is not consistent with current study. Neutrophils population was ranges 14-23% and there were no significant (P<0.05) changes from 0 day to 6th days which is similar with previous studies (Dang *et al*., 2009) [5]. Transition milk and mature milk almost shows similarity with a very little difference particularly last days transition milk. Present studied also conclude along with previous studies when somatic cell count increase in healthy mammary secretion, lymphocyte papulation decreases and when SCC papulation decrease lymphocyte papulation increases, vice versa (Schwarz *et al*., 2011) [35].

Viability (Live/dead) of cells:

Viability of the colostrum and transition milk SCC of different days KF cows have been presented in fig. 3. At the time of calving percentage of live was lowest and it was 42.2, which was increase significantly (P<0.05) on day 2, and further increased significantly during days 3, 4, 5, 6 and 7th day.

Udder health condition can be evaluated by the measuring live and dead cell count because its ratio vary with milking stage, mammary gland health status and milking interval. Paape *et al*, (2002) [27] reported the viability of non-infected mammary gland milk neutrophils was 63± 2% and after post inoculation of E coli, the viability of the viability of PMN isolated from milk of *E. coli*-infected quarters at 6, 12, 18 to 24, and 48 to 72 increased to 86, 93, 89, and 76%, respectively. Koewler *et al*., (2010) [16] studies in milk and reported cell viability was 39.5% for all cells and varied for each cell population from 26.7% for PMNs, to 32.6% for macrophages, and 58.3% for lymphocytes which is inconsistent with the present study of last day transition milk. Compare to previous study in current study of viable cells is slightly less in colostrum and transition milk with those reported for colostrum and milk by, Pertoft *et al*., 2000 [31]; Gonzalez *et al*., 2017 [12], studies. higher level of dead cells in day first colostrum may be due to phagocytosis of fat granules, higher viscosity and colostrogenesis start few weeks earlier leads to most of cells complete their life span at time of first milking.

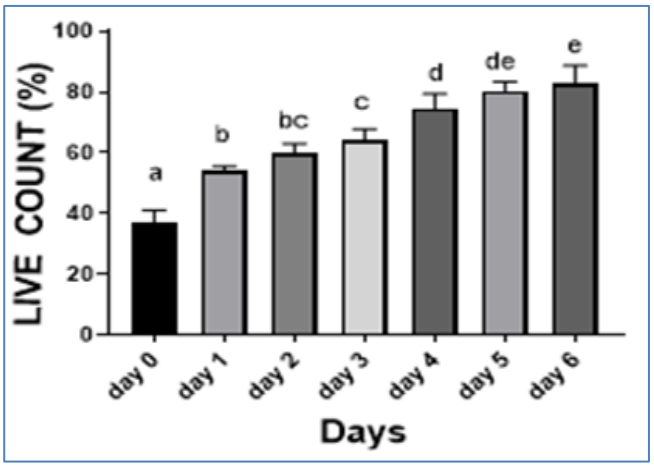

Fig 3: Viability of the colostrum and transition milk SCC in different days

Growth factor IGF1

The level of IGF1 were significantly (P<0.05) changes from day 0 to days 6, there were no significant difference between days 5 and 6 as shown in figure 4. Highest concentration was on the day 0 which mean value was 186.19 ng/ml and on the days 6 it was reaches at lowest level which mean was 33.85 ng/ml. Our results show that there is rapid and more significant decrease in IGF1 concentration in both colostrum and transition milk.

IGF1 is important growth factor present in colostrum and milk, in initial hour colostrum it is present in higher amount and decline substantial amount in time dependent manner. We measured 186.29 ng/ml in the first milking colostrum, then IGF1 concentration declined each 24 hrs milking and value on the days 6 was 33.85 nm/ml, the results correspond with earlier published values of IGF1 in bovine colostrum by Pakkanen and Aalto, 1997 [28]; Ginjala and Pakkanen, 1998 [11]; Elfstrand *et al.* 2002 [8]. Presence of higher amount may be due to its critical demands for calves after birth to full fil important functions like, metabolism of protein and carbohydrate, formation of blood vessels (angiogenesis), survivability, proliferation, growth and development of the GI-tract of newborn calves (Donovan and Odle, 1994 [7]; Pandey *et al.*, 2020 [29]).

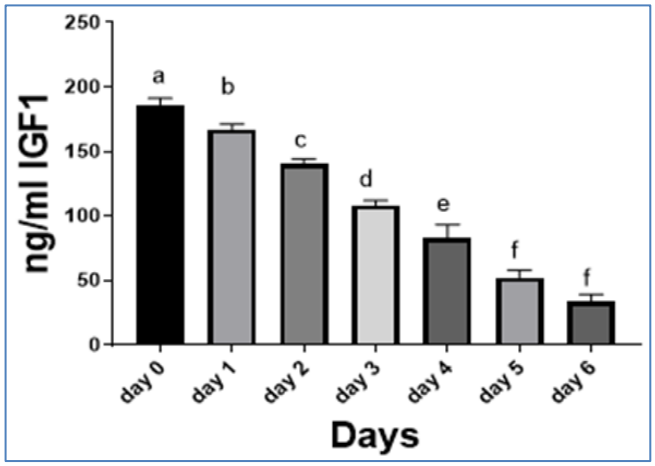

Fig 4: The level of IGF1 in the colostrum and transition milk

Conclusion

The present study result of SCC, DLC and live cells percentage of consequent days can be used as reference for early prediction and detection of mammary gland status and chance of occurrence of mastitis which accurse very frequently during early lactation, can be reduce the dairy production loss and increase the economic output. These assays provide the information, to specifically measure, the concentration of different cells and growth factors, which may also provide information about their specific biological functions in colostrum and transition milk.

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