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IDENTIFICATION OF CELLULAR CONSTITUENTS OF BREASTMILK: AN ANALYSIS

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Abstract
Recently, breastmilk has been observed to be an ideal source of stem cells. Here, different cellular constituents that are present in human breastmilk, identified by cytological staining procedures. Smears were prepared from breastmilk and fixed with ether and 95% ethyl alcohol (1:1) followed by staining with Giemsa, Hematoxylin and Eosin. Different cell types were observed. These were identified by their unique morphological patterns and archetypal geometry of the nucleus. Monocytes have metaphorically bean/ kidney-shaped or horse-shoe shaped or heart shaped nucleus whereas lymphocytes have large nucleus. Macrophages display irregular shape. Plasma cells have small, dense, eccentric nucleus, voluminous cytoplasm. Neutrophils, basophils and eosinophils were recognized by their lobulated nucleus. Lactocytes are characterized by exclusive lipid inclusions. There was a predominant occurrence of vacuolar space resembling morphologically to alveolar and lobular cells of functional mammary gland of human. The expression of the following cell types in mature human milk concludes that they have both hematopoietic and mammary origin. This study further illustrates the possibilities of breastmilk to consider as a unique cellular model that can be used to study breast pathology and biology of mammary glands.

Introduction

Detailed cellular analysis of any physiological products or tissues is an efficient way to study basic cell biology and associated disease pathology[1], Identification of cells and cell types often yields deep insights into novel functions of core cellular machinery and become a diagnostic tool deciphering physiological status of individual[2]. Differential staining is a classical technique used to visualize cellular morphology, structure and classify the bonafide cells or cell types present in tissues and body fluid[3]. Human milk is a dynamic physiological fluid that contains many nutritional components, growth factors, bioactive molecules and heterogeneous population of many cell types. There are three distinct stages of breastmilk: colostrum, transitional milk and mature milk[4].

Colostrum is produced in the early postpartum period. It contains a plethora of proteins, fat-soluble vitamins, minerals, immunoglobulins, various cellular components and growth factors. The detailed cellular composition of human colostrum is well defined[5,6]. Soon after parturition, the secretion of colostrum lasts for approximately two

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weeks, after which mature milk formation starts, which lasts till involution. So, the neonates mostly consume mature milk during lactation period and are exposed to dominant cell types present in it[7]. Recently, a study conducted to evaluate the leukocyte populations present in term and preterm breastmilk by using multicolour flow cytometry[8]. This method allows a robust measurement of an extended differential leukocyte count (up to 11 leukocytes subsets) in both healthy and diseased individuals. However, this high-throughput and high-content analysis system needs sophisticated instrument and expert personals to operate such instrument[9, 10]. On the other hand, preparation of cytological smears and application of different staining procedure provide a more comprehensive view of underlying tissue architecture and complete cellular morphology of concern tissues or physiological fluid[11]. The principal aim of this study is to analyze the detailed morphological pattern of different cells and cell types present in mature milk by applying different cytological stains.

Materials and methods

The study protocol was approved by the institutional human ethical committee. All participants were properly explained about the purpose of the study and their written consent was obtained prior to collection of sample. A total of four breastfeeding women (duration of lactation – four to 12 months) were recruited to participate in this study. Participants were asked to clean the nipple with soap and water before expressing milk. Breastmilk was expressed using electronic pumps, hand-operated pumps or manual expression (according to the donor's usual method). The milk was collected in SPECI-CAN (Romsons medicons, India) in the morning hours and immediately transported to laboratory in an ice bag and processed within four hours of expression.

Cellular evaluation

A drop of breastmilk was pipetted onto a glassslide and a coverslip (SSU Plain Glass Slides Cover Slips, India) was placed. Then microscopic examination was undertaken without staining the smear. For the cytological evaluation, the smears were prepared on separate clean glass slides by using a pasture pipette (Tarson, India). Then, the smears were fixed without drying by immersion in a solution of equal parts of ethers (Merck, USA) and 95% ethyl alcohol. The fixation procedure was adopted from Papanicolaou et al. (1958)[12]. Giemsa staining (Himedia, India) and haematoxylin and eosin (H and E) (Qualigens, Thermo Fisher Scientific, India) were employed for staining the smears to study the cellular morphology. For conducting H and E staining these slides were serially rehydrated and dehydrated in 30%, 50%, 70%, 90% and 100% alcohol, then stained with haematoxylin and eosin. Giemsa staining of the slides was done as per manufacture protocol. After removal of excess stain the detailed morphological analyses were carried out under compound microscope (Olympus, America) for identification of specific cell types.

Light microscopy and photomicrographs

Olympus CX-41(Olympus America, Center Valley, PA 18034-0610) equipped with Plan N 40×, 0.65 numerical aperture dry, and Plan N, 1.25 numerical aperture 100× oil objectives. Images were captured using a digital camera (Nikon, India) coupled to compound microscope. A total of 200 slides were examined.

Results

A heterogenous cell population was recorded when smears of pure breastmilk were observed through compound microscope prior to identification of cell types by staining. The cells of different shapes and sizes were found to uniformly distributed throughout these smears. Some were appeared small while others are big and cells having circular, elliptical and spherical morphology were clearly visible (Fig: 1 A). In the present investigation considerable number of foam cells (or *"cells of Donné"*) were observed in the smears of mature milk (Fig: 1 B). They were approximately circular and somewhat flattened. They had a well distinct border with large prominent nucleus and granular cytoplasm.

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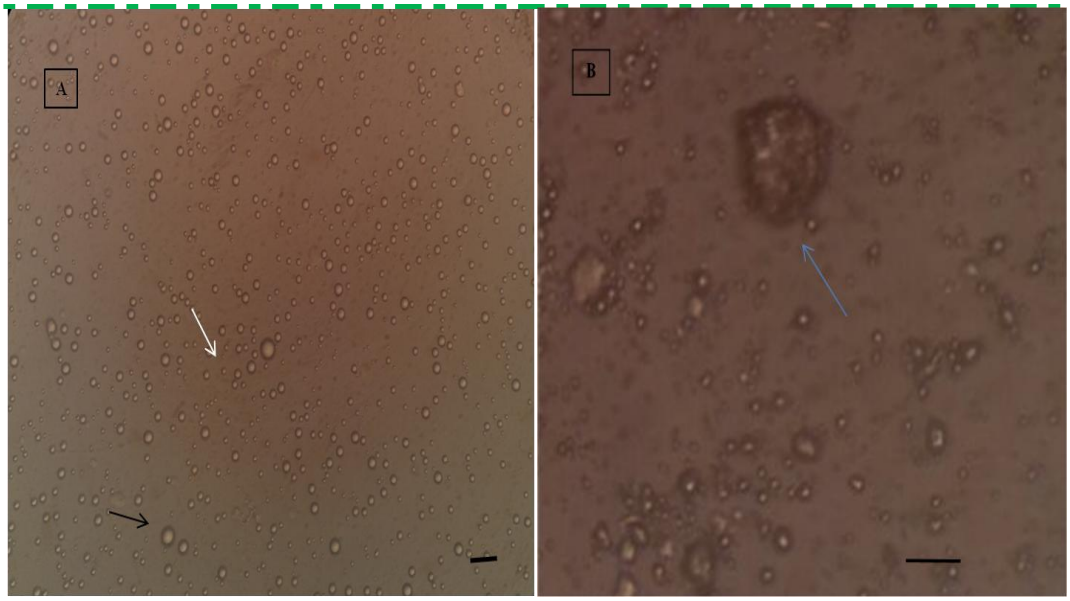

Fig: 1- Cells present in pure human milk without using any stain: A-Different shape and sized cells present in breastmilk (100 x magnification). White arrow indicates smaller size cells and black arrow indicates larger size cell; B- Typical foam cell (100 x magnification).

Monocyte lineages (Fig: 2A) having spherical and bulky shape were frequently observed in the smears. Sometime they had an irregular contour. They had characteristic horse-shoe shaped or heart shaped or kidney shaped nucleus which located at the periphery and show dark purple colour on staining. These were very much similar to the monocytes present in blood with an exception that milk monocytes contain numerous fat globules and have fewer granules in their cytoplasm. Further, microscopic analysis were observed the presence of irregular shaped macrophages having various interface. These were easily distinguished from other cells by their larger size and finely vacuolated cytoplasm filled with both small and large vacuoles (Fig: 2 B). Lymphocytes were round or spherical in shape. The nuclei were rounded in shape in both small and large lymphocytes. In both the lymphocytes the nuclei occupied almost the entire cell leaving a thin rim of light violet or almost transparent cytoplasm towards the periphery. Nuclei of both the lymphocytes were dip violet in Giemsa (Fig: 2 C, D).

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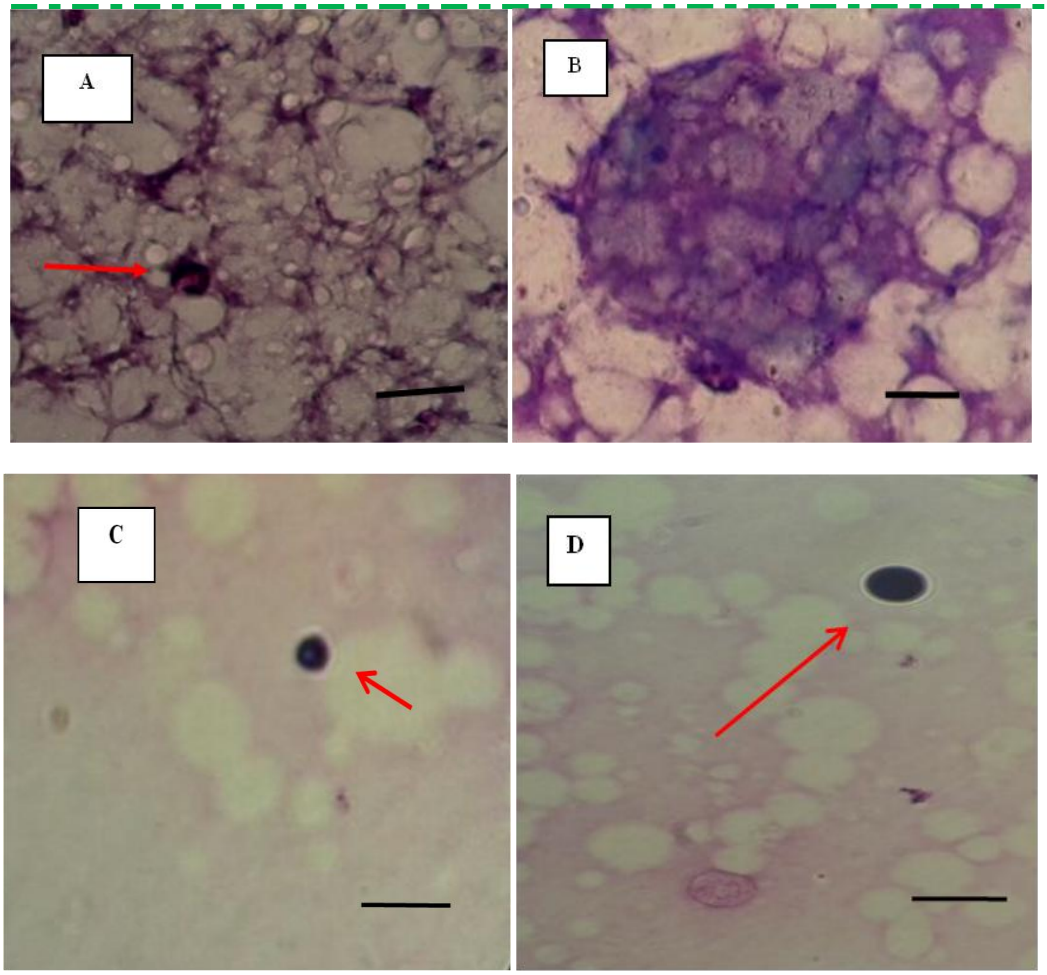

Fig: 2- Different types of agranulocytes present in breastmilk. A. Monocyte (Giemsa staining), B. Macrophage (H and E staining), C. Small lymphocyte (Giemsa staining), D. Large lymphocyte (Giemsa staining). Here all the cell types were viewed at 1000 x magnification. Arrow heads indicate respective cell type.

In the present study, a set of granulocytes were infrequently observed and identified by their morphological pattern and shape of the nucleus. Polymorphonuclear neutrophils were marked by their multi-lobed nucleus, these became dark and appeared dark blue or almost black on Giemsa staining (Fig: 3A). During the present investigation, the presences of basophils in the breastmilk were occasionally noticed (Fig: 3 B). Basophils were identified by the presence of U shaped, bi-lobed nucleus obscured with granules. These cytoplasmic granules became dark pink on staining with H and E staining. They had high nucleus to cytoplasm ratio. The presence of mast cells was also observed in few smears of breastmilk. These were identified by uni-lobular round nucleus and dense basophilic granules occupying all the cytoplasm (Fig: 3 C). Here, eosinophils were noticed, their bi-lobed nucleus appeared blue and cytoplasm contain large granules, enzymes and proteins appeared pink on H and E staining. It is invaluable to note these granulocytes were sparsely observed in milk smears, when collected from healthy dyad.

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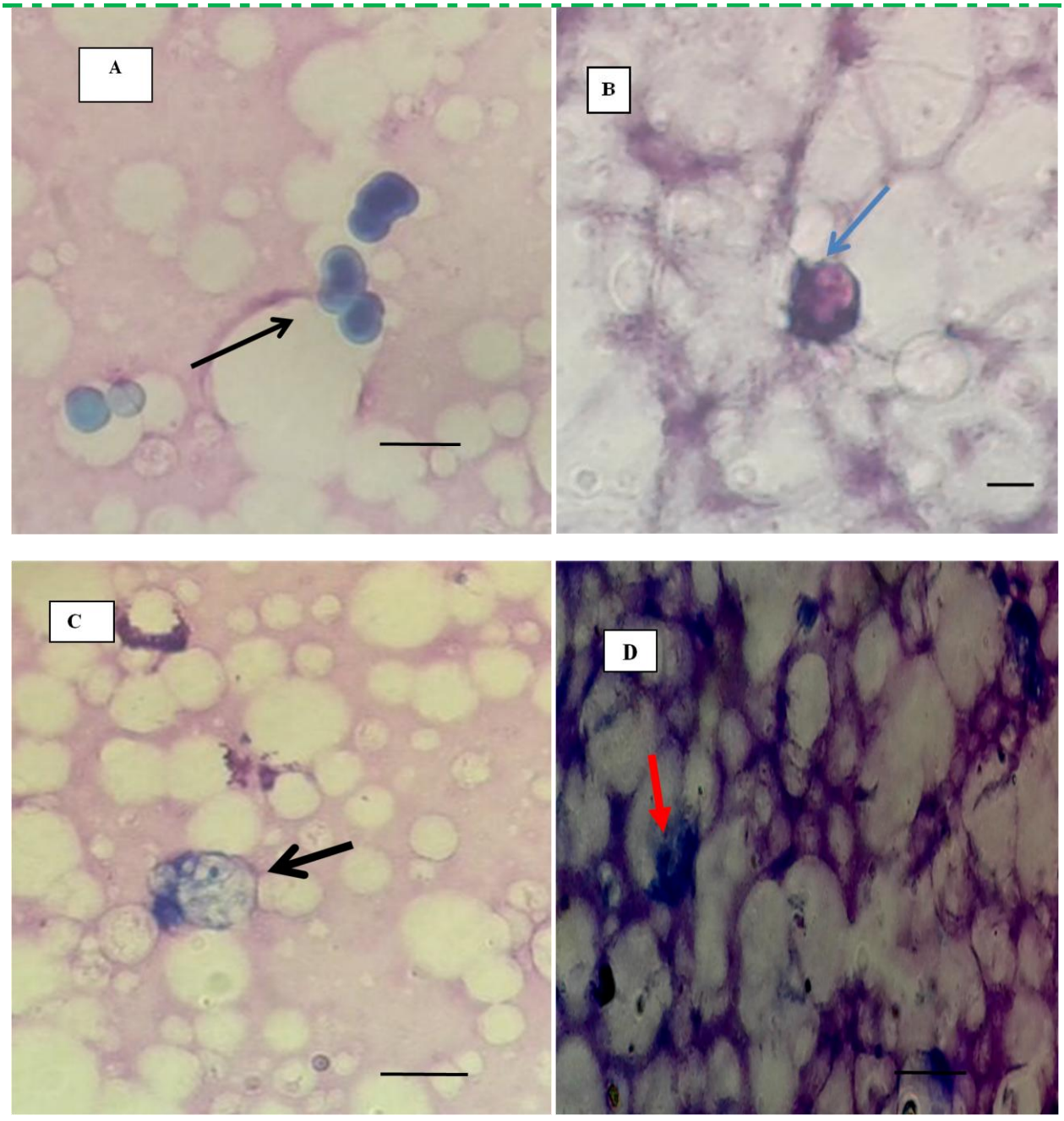

Fig: 3- Different types of granulocytes present in breast milk. A. Polymorphonuclear neutrophi,(Giemsa staining), B. Basophil (H and E staining), C. Mast cell (H and E staining), D. Eosinophil (H and E staining). Here all the cell types are viewed at 1000 x magnification. Arrow heads indicate respective cell type.

In the present investigation, plasma cells exhibit a small, dense, eccentric nucleus, voluminous cytoplasm were infrequently observed (Fig: 4 A). Lactocytes were observed predominantly and identified by their peculiar morphology of presence of invariable amount of fat inclusions or fat droplets (Fig: 4 B). The lobular cell clusters

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were invariably present in the breastmilk. These are identified by presence of vacuolar space for accumulation and storage of milk (Fig: 4 C). The parenchymatous alveolar cells having cuboidal morphology accompanied with many microvilli and fat droplets were also observed (Fig: 4 D). The results of this experimental observations can contributed efficiently to study diagnostic cytology, mammalogy, immunology and oncology of mammary gland and associated organs.

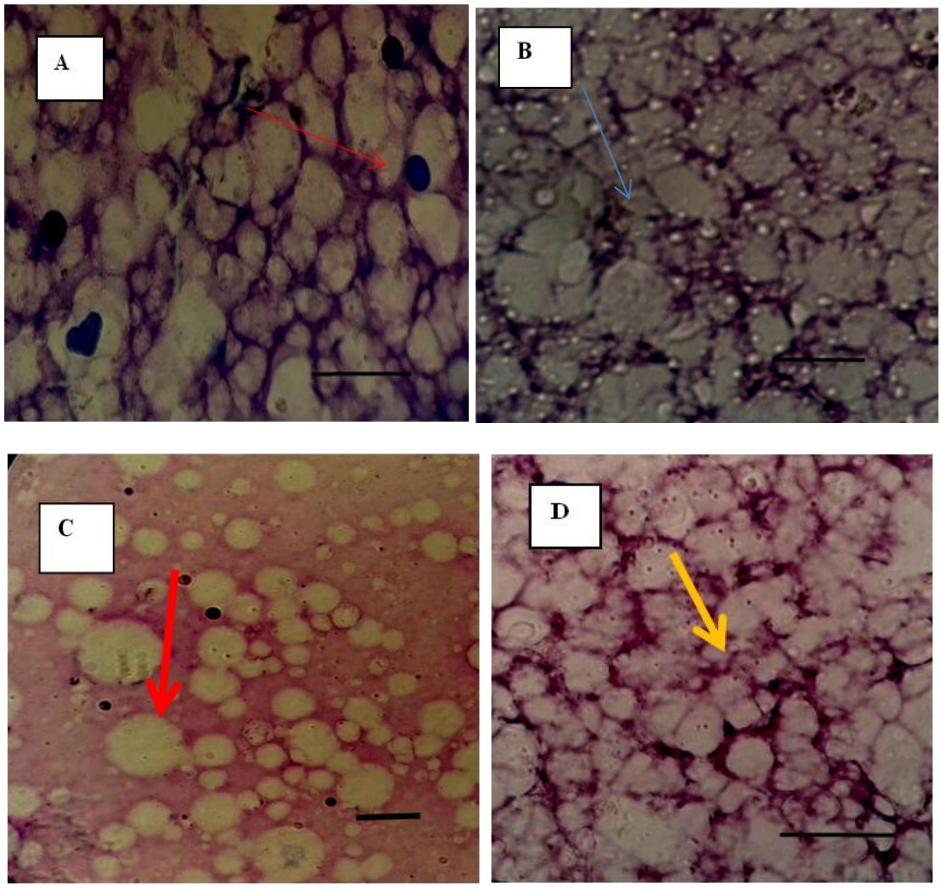

Fig: 4- Specific cell types present in breast milk. A. Plasma cell (H and E Staining), B. Lactocytes showing lacteals (Giemsa staining), C. lobular cells (Giemsa staining), D. Alveolar cells (Giemsa staining), Here all the cell types are viewed at 1000 x magnification. Arrow heads indicate respective cell type.

Discussion

During development, cells undergo change in behavior and gene expression that distinguish them from their neighbours and enable them to perform their assigned function[13, 14]. The cellular function attributed to different cell types is determined the shape of nucleus, cytoplasmic content and its structure[15]. This concept intrigues many researchers to reveal different cell types present at different allocated tissue systems[16, 17]. Most notably, after placental transmission the second mode of transfer of immunity occurs via the milk to newborn[18]. Composition of the human milk has indeed been a topic of research over the years. Recently it is reported that apart from the nutritional contents, the presence of various cellular components and numerous bioactive factors makes it invaluable source to study biology and pathology of mammary gland, this may open new therapeutic opportunities in cell-based therapies and may create novel pharmacological formulation to be used medicine and clinical biology[19]. The results of this analytical study illustrates breastmilk contains heterogeneous cell population composed of cells and cell types

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of various shape, size and kind. Previous reports also elucidate the complex nature of human milk, which has a heterogeneous composition of cells comprising of immune cells, epithelial cells, early-stage stem cells, progenitors and more differentiated cells[4, 20-22] The present investigation also demonstrates the variant patterns of cells and cell types in term of size, number and composition.

The morphological patterns and the somatic cell content of cells present in colostrum was well defined[6, 23]. The major cellular constituents of colostrum are colostral corpuscles, macrophages, small lymphocytes, neutrophils occasionally epithelial cells[5]. The origin and function of foam cells is the subject of considerable interest for academician, oncologists and clinical practitioners. According to some authors these are desquamated epithelial cells[24], while other contended that these may be macrophages from the blood[25, 26]. *"Cells of Donné* " or foam cells are invariable present in colostrum. However, accumulating evidences suggest these cells are universally present in normal, atypical and malignant mammary secretion[12, 27]. Similar to earlier publish reports in this study the presence of foam cells in breast milk is also demonstrated. In the present investigation, classical staining procedure is followed to identify different cells and cell types. Cell staining is a necessary and useful technique to visualize cell morphology and structure under a microscope. This cost effective conventional approach decipher detailed regarding particular cell types based on their phenotypic characters and structural integrity of nucleus. This technique has been widely used in many areas basic biology and medicine such as cytology, hematology, oncology, histology, virology, serology, microbiology, cell biology and immunochemistry[28]. Seminal studies have used this technique to evaluate exfoliative cytological smears of mammary secretion and screening of oral carcinoma[12, 29].

The nuclear shape within a given cell defines that particular cell type and it depends on the functional activities of that cell type[30]. Previously it is reported that biological processes performed by particular cell type such as proliferation, apoptosis, differentiation, development, and aging is require careful orchestrate spatial and temporal expression of gene[31]. Further, bidirectional trafficking of functional components like tRNAs and mRNAs, histones, DNA and RNA polymerases, gene regulatory proteins, and RNA-processing proteins between nucleus and cytoplasm is ascertain the shape, size of nucleus and nature of cytoplasm[32]. So, for proper identification of cells and cell types, the shape, size and position of nucleus and nature and texture cytoplasmic proteins can be taken as thorough reference. Human milk comprises of complex cellular hierarchy that originate from both haematopoitic stem cells (HSCs) derived blood cells and mammary stem cells derived breast-cells. Hematopoietic stem cell system (HSC) is a demand control system consists of a HSC niche, containing stem and progenitor cells and its fully differentiated progenitors reside in the bloodstream. The demand from the organism occurs via changes in the levels of differentiated blood cells, which feedback this demand to the primeval (stem and progenitor) cells[33]. The bi-potent mammary stem cells, which poorly present in the resting breast become activated and undergoes controlled program of proliferation and differentiation towards milk secretory cells during pregnancy and lactation[19]. In the present study, a set of agranulocytes, granulocytes and few specific cell types only limited to mammary gland were recorded in human milk. Moreover, health status of dyad also influence the cellular composition of breastmilk[34].

Monocytes and macrophages are active components of innate immune system[35]. These play major role in development, inflammation and anti-pathogen defense mechanisms either eliminating the foreign agents directly or by organizing different phases of inflammatory response[36]. Monocytes have bilobed nucleus[37]. The nucleus is frequently horse-shoe shaped or kidney shaped or heart shaped. The reason for formation of bi-lobed nucleus in monocytes is not yet fully understood. This may be due to the fact that it makes the cell flexible to pass through the basement membranes and enter to tissues with ease. The nucleus of monocytes generally acquires round shape following recruitment into tissues and on differentiation into a variety of macrophages and other cell types[38]. Macrophages are one of the most important professional phagocytes in the body, capable of killing a wide spectrum of foreign invaders using the active lysosomal system present in these cells. Most probably this functional competency attributes its irregular shape, as it has to undergo transendothelial migration to elicit its response. Irregular shaped macrophages with granules in their cytoplasm and round shape nucleus that were seen in the

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present investigation corroborate with the findings of the earlier publish report[39]. Notably, the macrophages of breastmilk show higher motility than their blood counterparts[40].

Lymphocytes appear to be the least interesting of all the leukocytes may be due to the monotonous sameness of appearance. As it gives no clue about its complex history, its present function or its future and nor could able to explain why T cells responsible for cellular immunity while B cells provide humoral immunity[41]. In the present study, there was a predominant occurrence of different lymphocytes. Small lymphocytes are non-DNA synthesizing cells. These are oligopotent stem cell and possess high nucleus to cytoplasm ratio[42]. Indeed, these small lymphocytes show no functional activity until they encounter antigen, which is necessary to trigger their proliferation and differentiation into large lymphocytes, B cells and T cells[32, 43]. Literature suggests these progenitors are larger in size having a larger nucleus and higher cytoplasmic content than small lymphocytes may be to perform specialized function providing innate and adaptive immunity[44].

In the present study, the presence of different granulocytes such as neutrophils, basophils, eosinophils and mast cells were less frequently recorded. The granulocytes present in human milk have similar morphology to that of granulocytes present in peripheral blood. Neutrophils form first line of defense by forming neutrophil extracellular traps, participating actively in oxygen dependent and oxygen independent phagocytosis process and secreting variety of biologically active compounds[45]. Basophils are non-phagocytic cells[46]. Basophils and mast cells share similar phenotypic and functional characteristics and express complementary and partially overlapping roles in acquired and innate immunity, including both effector and regulatory activities Bashophils and mast cells mainly concerned in pro-inflammatory responses to allergens and confer protection against pathogens[47]. The mast cells and basophils also release *histamine*, as well as smaller quantities of *bradykinin* and *serotonin*, heparin, slow-reacting substance of anaphylaxis, and a number of lysosomal enzymes. Granulocyte eosinophils contains chemical mediators, such as histamines and proteins such as eosinophil peroxidase, ribonuclease (RNase), deoxyribonucleases (DNase), lipase, plasminogen and major basic protein. Many appear to be degranulated to release mediators, granular proteins and superoxides and are thereby considered to play an important role in both parasite and host tissue damage[48]. Eosinophils migrate to inflamed areas and obliterate the foreign antigens by anti-parasitic and bactericidal activity or by extracellular trapping (ETs) mechanism. These are also participated in type-1 hypersensitivity reactions and modulate inflammatory responses[49].

Among the class of leukocytes the "granulocytes" are identified by their diversifying shape of nucleus[50]. The shape of the nucleus in neutrophils is multilobed, basophils and eosinophils have bilobed nucleus and whereas mast cells has round shaped nucleus. The shape of nucleus in neutrophils is may be due to the fact that it shows migration through even smaller than 1μm constrictions of the endothelium, either between or through cells. Accumulating evidences suggest lobular arrangement makes the nucleus easier to deform that facilitates the cells to pass through small gaps in the endothelium and extracellular matrix more easily[51].The formation of neutrophil extracellular traps (NETs) involves loss of structural configuration of nucleus. This allows de-condensation of chromatin, break down of nuclear and cell membranes, releasing NET into the extracellular space over ~1–4 h[52]. This particular mechanism "extracellular traps (ETs)" is also executed by other cell types including, mast cells[53], eosinophils[54] and macrophages/monocytes[55]. ETs consist of nuclear or mitochondrial DNA as a backbone with embedded antimicrobial peptides, histones, and cell-specific proteases and thereby provide a matrix to entrap and kill microbes and to induce the contact system. During this process there is a serious change in structural configuration of nucleus. This may be due the fact that it is easier to dissemble a lobulated nucleus. These alterations create this antimicrobial effector mechanism capable of snaring and killing a wide spectrum of microbes[56].

Lineage commitment toward granulopoiesis begins with the HSCs losing its ability to self-renew, and the stepwise acquisition of specific myeloid identity[57]. These are accompanied by unique changes in the shape and size of the cell, including dramatic changes leading to a multilobular nucleus in the final stage of granulopoiesis[58]. Although this unique morphological change has been extensively studied, but the exact mechanisms underlying it, are not yet

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fully elucidated. A recent work demonstrates shape of the neutrrophil nucleus may contributed by marked changes in its nuclear envelope protein composition[59]. The modulation in nuclear morphology of neutrophil is associated with almost complete loss of lamin A/C expression and increased expression of lamin B receptor (LBR) [60]. The lamin proteins provide structural support to the nucleus and protect against damage from mechanical stresses[37]. Particularly, the ratio of lamin A: B balances the stiffness of the nucleus against its elasticity[61]. The lower content of lamin A and C, with an increase in lamin B protein in nuclear envelope seems to influence nuclear shape. Another study also demonstrates that elevated level of LBR is necessary for the non-ovoid shape[51]. The granulocytes have lower lamin protein content than monocytes or macrophages which make their nucleus hyper-segmented. Another nuclear protein Esc1p also influences nuclear shape[62]. In response to microbial pathogens with the necessary specificity and rapidity, B cells are exquisitely regulated in the bone marrow and activated in the periphery[63]. Plasma cells are the terminally differentiated, non-dividing effector cells of the B-cell lineage. These cellular factories devoted to the task of synthesizing and secreting thousands of molecules of clonospecific antibody at each second. Experimental evidences suggest that there are two stages in the initiation of lactation- secretory differentiation and secretory activation. Secretory differentiation represents the stage of pregnancy when the mammary epithelial cells differentiate into lactocytes with the capacity to synthesize unique milk constituents such as lactose. Lactocytes are identified by presence of fat globules[34, 64].

In the present study, enormous number of lactoytes were observed in human milk. Secretory differentiation process requires the presence of a 'lactogenic hormone complex' of the reproductive hormones, estrogen, progesterone, prolactin and some metabolic hormones. Secretory activation on the other hand, is the initiation of copious milk secretion and is associated with major changes in the concentrations of many milk constituents. The withdrawal of progesterone triggers the onset of secretory activation where as the presence of prolactin, insulin and cortisol is essential[65, 66]. Lobules in breast tissue can be classified based on their degree of development into four different types namely Type-one, Type-two, Type-three and Type-four [67]. For the first time, in this study there was copious presence of Type-4 lobules in breastmilk was recorded, which are present only in the lactataing breast. During lactation, secretory cells synthesize milk components, which are collected in alveoli and duct lumen[68]. It is reported that the alveoli of the lactating breast are lined with a single layer of cells. Breast alveoli are balloon-like structures lined with milk-secreting cuboidal cells, or lactocytes that are surrounded by a net of contractile myoepithelial cells[69]. For the first time, the live alveolar cells of lactating breast, were observed in mature human milk.

Conclusion

As breast biopsy of lactating women is not medically recommended, so anatomical diagrams and descriptions of the gross anatomy of the lactating human breast are based on meticulous dissections of the breasts of lactating cadavers (Ramsay et al., 2005) which may not reproduce complete cellular hierarchy of lactating mammary gland. In mature breastmilk, morphologically complex architecture of lactating mammary gland is reflected. The cellular profiles of mature milk are closely resembled with different cell types present in blood and colostrum, so may give new dimensions to medical research as it can be used for diagnostic purposes to study the divesting pathology of aberrant breast, help us to understand biology of mammary gland. The mature human milk may be treated as a new drug which helps to rejuvenate, replenish and repair the disease tissue in adults. In future, breastmilk may be consider as a novel formulation that can delay the age-related diseases, slow the process of aging and thus prolong human life.

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Conflict of interest

The authors do not have any conflict of interest

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