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Problems in Breastmilk Cell Isolation

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Abstract: Background: Breastmilk contains a plethora of bioactive factors, including biochemical and cellular components. Maternal cells in breastmilk comprise a heterogeneous population of epithelial cells, leukocytes, progenitor and stem cells that dynamically respond to maternal and infant needs. Previous studies have confirmed the presence of many types of cells in the human breastmilk cells. Breastmilk cells is susceptible and only appear in small number in the breastmilk. Although cellular analyses have been previously conducted in breastmilk, optimization of the methodology to isolate and process cells from breastmilk is lacking. In this study, we aim to elucidate the problems in breastmilk cell isolation using two culture media. Methods: We isolate human breastmilk cell using two different media, DMEM with fetal bovine serum (FBS) and MammocultTM. Human breastmilk was prepared and processed using protocol from previous study and then cultured into two different media. Results: We faced some problems in breastmilk cell isolation process with two media. Breastmilk sample preparation, reagents use, and time of isolation process is major factor which determine cell viability in breastmilk cell culture. Conclusion: Error in breastmilk sample preparation, time consuming isolation process, and breastmilk sample volume may affect the breastmilk cell viability.

Key words: breastmilk cell, isolation, cell culture.

1. Introduction

Breastmilk contains a plethora of bioactive factors, including biochemical and cellular components that provide a multitude of nutritional, immunological and developmental benefits to the infant. [1] Milk

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composition can be varied. It differs among mammalian species, and many factors may be associated with this variation, including the maternal itself, environment, and potentially genetic factors. [1, 2] Intraindividual factors also contribute to this variation, such as the stage of lactation, the degree of breast fullness, infant feeding, the health status of the breastfeeding dyad, and other factors. [2, 3, 4]

Mother's milk can be distinguished between nutritional and bioactive or biochemical components. The latter are growth, immunological factors, and cellular components. Typically, breastmilk is thought to contain epithelial cells and immune cells. [4] Most of the milk compositional research has been centered on its biochemical composition. [1, 2, 4, 5] In contrast, very little is known about breastmilk cells. This finding has perpetuated the thought that the leukocytes is the predominant cell type in human milk. [3] Although, leukocytes are abundant in colostrum, recent analyses using state-of-the-art techniques of cell characterization are revealing that leukocytes constitute the minority of cells (about ≤2%) in mature human milk when both the mother and infant are healthy. [3] Periods of infection, however, stimulate a rapid increase in breastmilk immune cell numbers, returning to low baseline levels upon recovery. [1, 3] This observation highlighted that during healthy periods, the majority of cells in human milk are of non-immune origin. [3, 4]

The majority of cells in mature human milk throughout lactation are epithelial cells such as lactocytes, and to a lesser extent myoepithelial cells based on flow cytometric analyses. [4] Luminal and the myoepithelial cells are thought to constitute up to <98% of the cells in human milk under healthy conditions. [4, 6, 7] Recent analyses have shown that breastmilk is more heterogeneous and that it also contains stem and progenitor cells. [2, 4] On top of that, breastmilk is also a source of commensal and beneficial bacteria (lactic acid and bifidobacterial). [1, 4] Some of these stem cells are known as part of mammary stem cells, being able to turn into functional lactocytes synthesizing milk in culture. [2, 5] Mammary stem cells [with the known marker profile cluster determinant (CD) $49f^+$ /$CD29^+$ /CD24low /cytokeratin (CK)5^+] are multipotent cells thought to prevail in the basal ductal layer in the resting breast, with the ability to self-renew and differentiate through a line of progenitor cells into the 2 main types of mammary epithelial cells: the luminal cells (known to express CK18) and the basal myoepithelial cells [known to express CK14 and smooth muscle actin (SMA)]. [6, 8]

Interestingly, subpopulations of breastmilk stem cells able to turn into cells from all three germinal layers, including neurons, glia, hepatocytes, pancreatic cells, cardiomyocytes, osteoblasts, chondrocytes, and adipocytes. [2, 8] They were also shown to express embryonic stem cell genes controlling self-renewal and differentiation, potentially facilitating the multi-lineage properties of these cells. Study of the human resting and lactating mammary gland showed that these cells exist in the lactating mammary epithelium, being scarce in the resting breast. [2, 6]

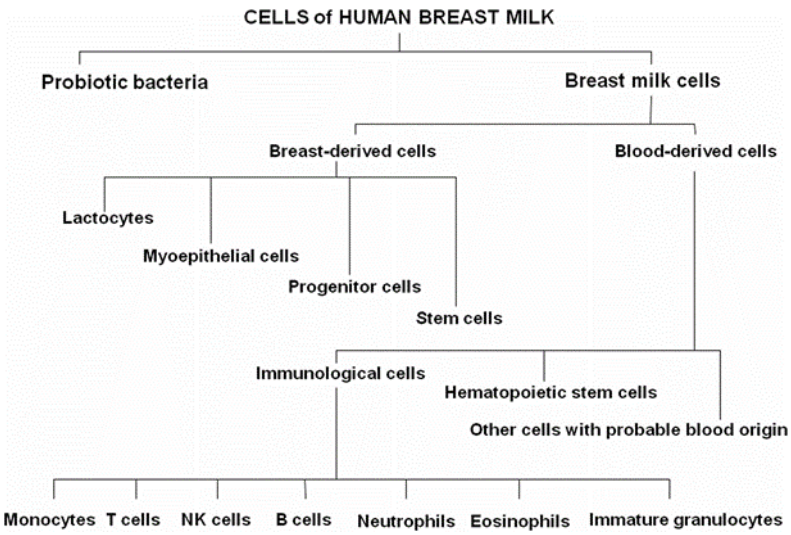

Fig.1 The current finding of the cellular composition of human milk. [8]

Previous studies have confirmed the presence of many types of cells in the human breastmilk cells, including the presence of stem cells. In 2007, Cregan et al. presented the first evidence that breastmilk contains stem and progenitor cells. [9] They showed that cell colonies established in culture from brestmilk contained cells positive for the mammary stem cell marker CK5 and the general stem cell marker nestin. [9] The ex vivo presence of $CK5^+$ and $nestin^+$ cells in breastmilk was confirmed by Fan et al (2010). [10]

In 2012, Hassiotou et al. released the first report demonstrating expression of pluripotency markers by cell sub-populations in breastmilk, and providing new data on the properties and origin of these cells, which were named human breastmilk stem cells (hBSCs). [11] BSCs not only self-renew in culture but also express pluripotency genes including the core transcription factors OCT4, SOX2 and NANOG and downstream targets KLF4, REX1 and GDF3. [11] Previous studies have been demonstrated that the lactating breast and breastmilk contain stem cells that have the ability to differentiate in culture into not only breast cells but also into bone cells, joint cells, neurons, glia, pancreatic cells. [11, 12] Twigger et al. (2015) found that the mammary gland is more mature in mothers of term infants, containing more cells with progenitor properties. In contrast, for mothers of preterm infants, the levels of the stem cell marker nestin were much lower. [6] These data suggested that breast milk stem cells may be involved in mammary gland function, lactation performance, and/or infant development. [6] Abd Allah et al. (2016) isolated MSCs from breastmilk of lactating rabbits. [7] It has been described that rabbit MSCs (rbMSCs) shows cellular and tissue physiology that closely resemble human MSCs (hMSCs). [7] Last study by Kaingade et al (2016) found that some growth factors was secreted by human breastmilk mesenchymal stem cells. [13] In conclusion, their results confirmed the presence of MSC-like population in the breastmilk which have ability to secreted some growth factors.

Breastmilk stem cells (BSCs) can be accessed noninvasively, ethically and sustainably and create no tumors. In addition, it is susceptible and only appear in small number in the breastmilk. [11, 12] Although cellular analyses have been previously conducted in breastmilk, [9, 10, 11, 12, 13] optimization of the methodology to isolate and process cells from breastmilk is lacking. In this study, we aim to elucidate the problems in breastmilk cell isolation using two culture media and may find the better approach to culture the breastmilk stem cells. Thus, we can convey breastmilk as a source of stem cells for regenerative medicine.

2. Materials and Methods

This study was performed at Physiology Laboratory, Faculty of Medicine, Gadjah Mada University Yogyakarta and all participants provided informed written consent. Healthy breastfeeding women (6) were recruited in Surakarta and Yogyakarta, covering a wide range of lactation stages, from month 1 to year 2, through one or multiple children. Pump-expressed mature breastmilk (50 ml) was obtained from each participant under aseptic conditions. The samples were collected in sterile 50 ml conical tubes and was transported to the laboratory immediately (<3 hours) upon expression under aseptic conditions.

Breastmilk cell isolation was using following procedure. Breastmilk was diluted 1:1 with Phosphate-buffered saline (PBS) in 50 ml conical tube and centrifuged at 3000 rpm for 20 min. The fat layer and liquid part skim milk were removed, and the cell pellet was resuspended in 4 ml PBS. The cell pellet in 50 ml conical tube was re-centrifuged at 3000 rpm for 10 min and resuspended in 4 ml PBS. Repeat the centrifugation process for 10 min at 3000 rpm. Those procedures were kept at 20^{o} C.

The final cell pellet was resuspended in 3-5 ml complete culture medium. We used DMEM with fetal bovine serum (FBS) and MammoCultTM as our medium. The first medium for isolation was DMEM with fetal bovine serum (FBS) which contains DMEM, Fetal Bovine Serum 10%, 2% Penicillin/Streptomycin, and 0,5% fungizone. The second medium was using MammoCultTM which contains MammoCult™ basal medium, MammoCult™ human proliferation supplement 10%, heparin (4 μg/mL), hydrocortisone (0.48 μg/mL), 2% Penicillin/Streptomycin, and 0,5% fungizone.

Lastly, breastmilk cells were seeded on both media and incubated at 37^{o}C and 5% CO_2, with daily media changes. After day 7, for passaging of adherent cells, the cells were trypsinized and only the live cells were considered for data analyses. The spectrometer is used to measure those cells.

3. Results and Discussion

We found that not all of breastmilk cells grow well on both media because of bacterial contamination. Even before we changed the media into the new one, the plates were already soiled. We tried remedial management of suspected infection with discarding a potentially infected culture, but the contamination still persisted. In the media plate, we just found 1-3 cells/HPF (Fig. 2). Trypsinization method was being used to separate the cells, but not succeed because the cells could not be released. Cells calculation process also could not be done because the amount of cell was too little.

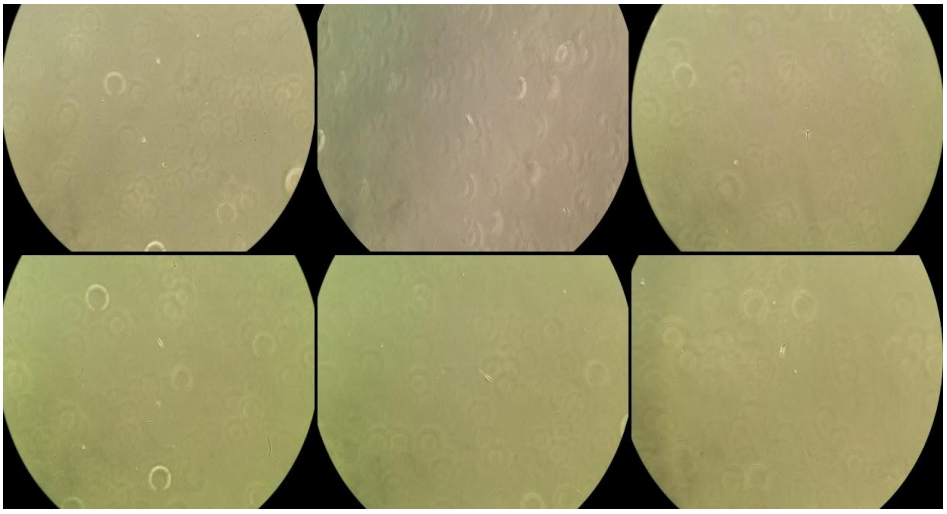

Fig. 2 Breastmilk cells did not grow well because of bacterial contamination.

We also found that two medium of cell have different effect in affecting cell growth. MammoCult™ medium (Fig.3) has more number of cells than DMEM+FBS medium (Fig.4). It may results from different type of proliferation supplement, MammoCult™ using human origin proliferation supplement and the other one using bovine origin proliferation supplement.

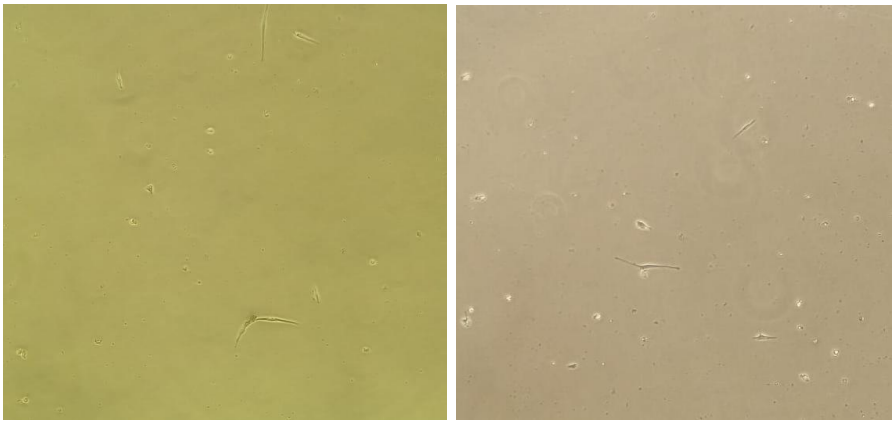

Fig. 3 Cell culture in DMEM + FBS medium

Problems in Breastmilk Cell Isolation

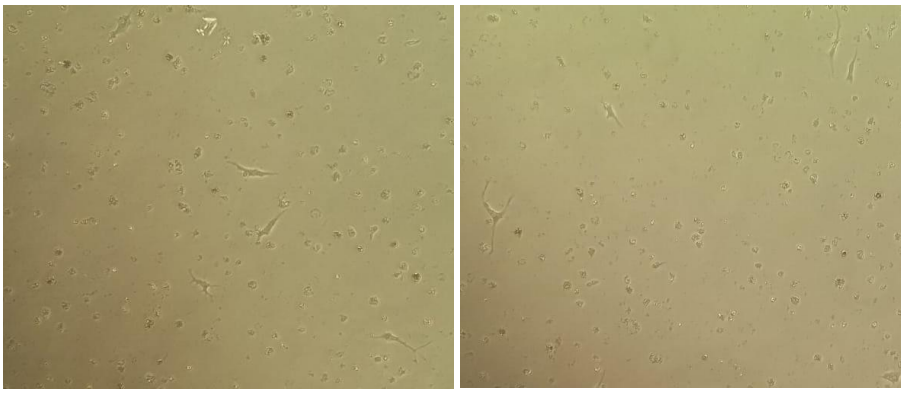

Fig. 4 Cell culture in Mammocult^{TM} Medium

Microbial contamination is a major issue in cell culture. Contamination may arise from samples, reagents, the operator and the laboratory environment, or from other cells used in the laboratory. We assumed that unsterile breastmilk sample preparation caused bacterial growth instead of breastmilk cells. Although we tried to collect sample under aseptic condition from breastfeeding mother, there were some mothers that pumped the milk at home and used sterile breastmilk plastic container instead at the laboratory. The total amount of the samples were too insuficient and sources of the breastmilk that came from different mother also could be an issue. We consider 1-2 mothers whom will become the sample in the next study.

Reagents use and time of isolation also became other major concern. The culture dish contains nutrients within the media that cells need to thrive and prosper. The wrong handling of the reagent or medium kit during the study could alter the nutrition of the media so cells could not grow. Unwanted infiltrators sneak into the cultures and endanger the balance of the system, spoiling the scientific results. This is why standard cell culture protocols often include the prophylactic use of antibiotics, such as penicillin, streptomycin, gentamicin or amphotericin as media supplements to lower infection rates. However, we only know little about the effects of these elements on the metabolism of cultured cells, cell proliferation, differentiation or gene expression. Stacey (2011) stated that most primary or normal human cell show reduced growth rates in the presence of antibiotics. The main goal of using antibiotics in cell culture is to kill or inhibit bacterial growth. On the other hand, we also acknowledged that antibiotics can deeply influence cell metabolism and harm the cells.

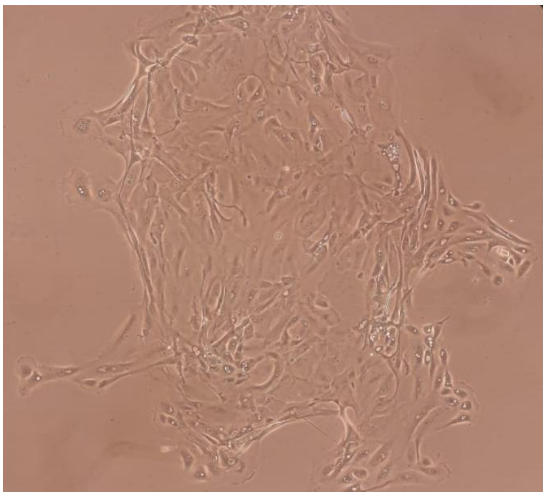

Fig. 5 Cells culture after optimization (Using Mammocult^{TM} Medium + FBS 10%)

At the final, after optimization process we found that cells were growth confluent (Fig. 5). We added 10% of FBS to Mammocult^{TM} as cell culture medium. We also found that breastmilk isolation time process was the key point to manage bacterial contamination and cell availability. A faster breastmilk isolation time was conducted by decreasing time for sample delivery. The breastmilk isolation was conducted no more than 2 hours. The total sample processing time (until cells was cultured) was no more than 4 hours. We also note that adequate volume of breastmilk sample was also a key point to get the good cell availability. We used at least 200 ml breastmilk to process cell isolation.

Avoiding bacterial contamination can be achieved with proper knowledge of good laboratory practice. Aseptic techniques, including a sterile work environment, sterile reagents and media, good personal hygiene and sterile handling, become a barrier between bacteria in the environment and the sterile cell culture. Fast breasmilk isolation process and adequate volume of breastmilk sample are a key point to get the good cell availability. Attention to laboratory layout, cleaning and maintenance, and quality control are also important factors in preventing contamination in cell culture laboratories.

4. Conclusion

To sum up, we faced some problems in breastmilk cell isolation and culture. We failed to grow breastmilk cells in this study due to contamination. Cells growth depends on aseptic condition during the study. Breastmilk sample collection has to be done under aseptic technique. Both the quality and quantity of the samples have to be considered. Other factor which may affects cell growth is media and proliferation supplement. Then, a laboratory environment, reagents, and media must be sterile to prevent any contamination.

The use of antibiotics as a prophylactic is relatable, but we have to be cautious. Antibiotics can kill the bacteria, but also harm the cell itself. Fast breasmilk isolation process and adequate volume of breastmilk sample are a key point to get the good cell availability. Error in breastmilk sample preparation, time consuming isolation process, and breastmilk sample volume may affect the breastmilk cell viability.

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