Review article

Isolation and culture methods of human breastmilk stem cells: A systematic review

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ABSTRACT

With the rapid development of cell-based therapy, the source of stem cells derived from non-invasive techniques have been intensively studied for their potential to replace the invasive source of stem cells. One of the alternative sources of stem cells is the cells taken from human breastmilk. Human breastmilk is always readily available, easy to access and can last for an extended period. Unfortunately, the standard protocol to isolate the cells from breastmilk is still not well explained. Our study aimed to investigate three main questions in human breastmilk stem cells: the optimal lactation stage, isolation and culture method, and the characterization of human breastmilk stem cells. We searched *in vitro* studies that used human breastmilk as a cell source of stem cells and clearly explained the step-by-step procedure. From the initial 457 studies, 15 articles fulfilled the criteria and were further analyzed. The results showed that the stem cells could be taken from breastmilk in any lactation stage and isolated with various techniques to obtain both pluri- and multipotent stem cells. Although breastmilk can be used as an alternative for noninvasive source of stem cell, the results of few study groups were found sometimes contradicting another group, resulting in confusing theory. Therefore, the standard protocol is still needed to tackle the confounding results.

Keywords: Breastmilk stem cells; isolation protocol; culture method

INTRODUCTION

With the massive development in regenerative medicine, many researchers are now turning to stem cells research. Stem Cells (SCs) is sometimes called the "mother of all cells" because of their self-renewing and differentiation into new cells capability (1). The extensive use of SCs is based on the fact that these cells can act as influential sources for learning and treating many clinical diseases. Among the hierarchy of SCs, at least two types are commonly used. The pluripotent stem cells that are capable of immense self-renewal was taken from either embryonic stem cells (ESCs), the inner mass of pre-implantation blastocyte, or by reprogramming back the somatic cells known as induced pluripotent stem cells (iPSCs) (2). Another type is the multipotent stem cells that can develop into cells from one similar germinal ancestor. Multipotent cells have a more limited differentiation capacity. Still, they are easily obtained from almost all somatic tissues in the human body, such as bone marrow, umbilical cord, adipose, skeletal and placenta (3,4). Among them are hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs).

Human breastmilk has been known for ages as an important source for baby nutrition. Recently, the human breastmilk stem cells (hBSCs) taken from the mammary epithelium and breastmilk has been the focus of many studies as they were found to be source of multiple SCs from different lineages (5). The hBSCs has the ability to produce a range of different cell types and can be taken in a non-invasive procedure. Thus, it has a great potential to be a valuable source for clinical research and applications.

The attractive side of SCs for therapeutic alternatives is due to their relative abundance and differentiation capabilities. Although an ideal stem cell source has yet to be recognized, the use of hBSCs as a non-invasive source of stem cells is promising. Many studies have explained the SCs population extensively, amid the large variations in the different cell types, isolation method, differentiation prospective, and marker expressions. However, to use SCs extensively in research or therapeutic application, the cells need to be expanded under robust *in vitro* conditions in a specific environment to maintain self-renewal and their pluri/multipotency potentials (6).

Therefore, the technique to obtain hBSCs is vital to understand. This review includes the questions that need to tackle when faced with the option of using breastmilk as a source of stem cells and seeks to highlight the differences among the lactation stage, isolation and culture procedures, and the marker expressions.

MATERIALS AND METHODS

The systematic review was conducted by searching relevant articles in PubMed following the PRISMA-P protocol for systematic reviews and meta-analyses using keywords as shown in Table 1. Pertinent additional articles through independent search efforts were included to ensure a thorough review. The inclusion criteria were all free accessible English literature published within the last 15 years (2006-2021), used human breastmilk stem cells, and explained clearly the *in vitro* method used in the articles. Duplicate articles, review articles and articles that did not use the stem cells component of human breastmilk were removed. The aim of this review was to find answers from the data extracted from literatures following the questions:

1. Does the lactation stage affect the number of SCs in human breast milk?
2. What kind of isolation methods were used to extract the hBSCs?
3. How are human breastmilk stem cells characterized and differentiated?

To avoid the risk of bias, the literature searching was performed by three independent reviewers. The data extracted by the first reviewer was thoroughly evaluated by a second reviewer and cross-checked by the third reviewer.

Table 1: Number of articles identified in PubMed using the keywords

Keywords	Articles found
Breast milk, human, culture	423
Stem cells in human breast milk	333
Breast milk, human, stem cells	40
Breastmilk, human, stem cells	15
Total	457

RESULTS

Based on the keywords, 457 articles were found initially, of which 440 were excluded because of the irrelevance title and abstract. Three articles were removed after a thorough review as they did not fit the inclusion criteria. Finally, only 14 articles matched all of the criteria. With the addition of one relevant article obtained from a manual hand search, the data from 15 studies were extracted.

The articles were then analyzed based on three research questions regarding the lactation stage, isolation and culture method, and the characterization and differentiation of stem cells from human breastmilk.

1. Does the lactation stage affect the number of SCs in human breast milk?

All but three articles studied mentioned the age of the human milk sample they used (Table 2), whether it is colostrum (the first milk released within the first week after birth) or mature breastmilk. Based on the stage of lactation, three articles used samples from colostrum (7–9), four articles used mature breastmilk (10–13), while five articles used both colostrum and mature breastmilk (14–18), the number of cells extracted varied in each sample. Although the average of all cells isolated was higher in the colostrum sample than mature breastmilk, it reveals that the number of stem cells is not affected by the lactation stage.

2. What kind of isolation method were used to extract the hBSCs?

Including in this study are the articles that explained the methodology applied for the isolation of stem cells from human breastmilk (Table 3). As the study of human breastmilk stem cells is still in a novel stage, it is not surprising that many different methods are used to harvest the human breastmilk stem cells.

Table 2: The summary of stage lactation as source of hBSCs

Lactation stage	Sample used	The average number of cells extracted	References
Colostrum	15 mL	$1.3x10^6$ to $3x10^6$ cells/mL	(7-9,14-18)
Transitional/mature breastmilk	5-200 mL	$1x10^3$ to $8x10^5$ cells per mL of milk	(10-13)
Not clearly specified	-	-	(19-21)

Table 3: The isolating method to extract hBSCs

Cell isolation	Washing method	Cocktail media	Reference
Directly centrifuged	Not explained	Roswell Park Memorial Institute (RPMI), *Fetal bovine serum* (FBS), insulin, hydrocortisone, *epidermal growth factor* (EGF), cholera toxin, penicillin, streptomycin, and fungizone	(12)
Directly centrifuged	Three times with EGF in RPMI	Serum-free mammary epithelial growth medium, B27, EGF, *basic fibroblast growth factor* (bFGF), heparin	(11)
Diluted 1:2 with Dulbecco's Modified Eagle Medium (DMEM) containing antibiotic	Washed twice with phosphate-buffered saline (PBS).	DMEM, heat inactivated human umbilical cord blood serum (hUCBS), penicillin, streptomycin. Medium was changed every 48hour	(8)
Diluted 1:2 in PBS	Washed once with PBS	RPMI, FBS, hydrocortisone, insulin, EGF, cholera toxin and penicillin- streptomycin	(19)
Diluted with 1:1 PBS	Washed three times with PBS	PBS, FBS Daily media changes after day 5	(13)
Diluted with DMEM	Not explained	The cell pellet was analysed without culture.	(7)
Diluted in PBS 1:1	Washed and resuspended in 10% FBS in PBS	The pellet was cultured in plates coated with gelatine. The media were changed daily.	(10)
Diluted in 1:2 DMEM containing antibiotic	Wash with PBS	DMEM, hUCBS, penicillin, streptomycin Medium was changed every 48 hours	(9)
Diluted 1:2 with DMEM	Washed twice with DMEM, FBS	DMEM/F12, knock-out serum replacement (KOSR), L-glutamine, penicillin, streptomycin, FGF, Non-Essential Amino Acid (NEAA), fungizone, b-Mercaptoethanol. The culture medium was refreshed every 24h	(16)
Directly centrifuged	Wash once or twice with PBS	Directly stained without culture	(15,17,
Diluted 1:2 with DMEM containing antibiotic	Washed twice with PBS	DMEM, FBS, penicillin-streptomycin-Amphotericin B. The Medium were changed every 48 hr	18)
Diluted with PBS 1:1	Not explained	DMEM, FBS, NEAA, EGF, bFGF, penicillin/streptomycin DMEM, KOSR, NEAA, EGF, bFGF, penicillin/streptomycin; The culture media were changed every 3 days	(20)
Diluted 1:1 with PBS	The pellets were washed thrice with PBS	DMEM/F12, FBS, glutamine.	(14)

To reduce any unwanted variable, all breastmilk samples were collected in aseptic condition and processed immediately within 2-4 hours after the extraction. The cell pellet was separated by directly centrifuging the breastmilk (11,12,15,17,18) and diluting in equal volume of PBS (10,13,14,19,21) or media (7-9,16,20). The centrifugation speed and duration to isolate the cell pellet varied from 300 to 2000 rpm for an average time of 10-20 minutes, with almost all the articles specifying the use of PBS to wash the cells. The cell concentration obtained ranged from 1.6 to 13 million cells/mL of breastmilk. Only four articles processed the hBSCs directly from the cell pellet without seeding the cells, while the rest of the articles cultured the cells in various mediums and growth factors.

There are several types of culture media and growth agents employed. The most often utilized medium is basal media DMEM or complex media RPMI. The medium was supplemented with various concentrations and combinations of growth factors and antibiotics. Most growth factors used are FBS, EGF, and bFGF. Nearly all articles used Pen-Strep as antibiotics, and only two add fungizone for antimycotic. The difference in culture protocol showed the range of days to change the medium between every day to every 5 days, and all the studies used the early passage of cell culture to gain further data.

3. How are the human breast milk stem cells characterized and differntiated?

The characterization of human breastmilk stem cells is summarized in Table 4. After isolation of stem cells, the characteristic and differentiation process were observed using various markers and methods. Among the immune phenotyping and genetic characteristic of hBSCs are hematopoietic, embryonal, mesenchymal and neuroepithelial markers. The hBSCs also proved to have multipotency as shown by the ability to differentiate into hepatogenic, neurogenic, adipogenic, osteogenic and chondrogenic lineages.

Table 4: The characterization and differentiation of hBSCs

Identification method	Cell marker	Differentiation	References
RT-PCR, Immunofluorescence	Nestin^{+}, CK14, CK18, CK19, CK5.	Not performed	(12)
RT-PCR, Flowcytometry, FACS	HSCs (CD34, and CD 133), MSCs (Stro-1) and neuro-epithelial lineages (nestin, CK5)	Not performed	(11)
Immunocytochemistry, Flowcytometry	MSCs (SMA, Vimentin, CD44, CD29, SCA1)	Adipogenic, chondrogenic and osteogenic	(8)
Immunofluorescence, ELISA	P63, sigma, CK14 dan CK18	Mammary lineage	(19)
Flowcytometry, qRT-PCR, WB	ESCs (OCT4, SOX2, NANOG, SSEA4, and TRA-1-60/TRA-1-81)	Adipogenic, chondrogenic, osteogenic, mammary, hepatocyte, cardiomyocyte	(13)
Flowcytometry	HSCs (CD34, CD133, CD117), MSCs (CD 90, CD 15, CD 73), myoepithelial cells, immune cells, adhesion molecules and Platelet-derived growth factor (PDGF)	Not performed	(7)
Immunocytochemistry	ESCs (OCT4, SOX2, NANOG) and MSCs (CD44, CD106, CD133)	Neurogenic	(10)
Flowcytometry	MSCs (CD 105, CD44, CD 177 and CD 140b)	Not performed	(9)
Differentiation	-	Hepatogenic differentiation	(16)
qRT-PCR, Flowcytometry	Nestin, SSEA4, TRA-1-60, OCT4, CD90, CD73, CD29, CD117, CD105, CD44, CD34, and CD 45.	Not performed	(17)
Flowcytometry, qRT-PCR	HSCs (CD34+), MSCs (CD44+, CD90+, CD105+) and pluripotent stem cell markers (SOX2, but not NANOG or OCT4)	Not performed	(18)
Flowcytometry	MSCs (CD 105, CD 90) Negative for HSCs (CD 34, CD45)	Not performed	(20)
Flowcytometry	MSCs (CD44, CD73, CD90, Sca1), ESCs (SOX2, OCT4, NANOG) and HSCs (CD34, Cd45, CD117)	Not performed	(14)
Flowcytometry	Negative for HSCs (CD34) but positive for MSCs (CD44 and CD 105)	Not performed	(21)
Flowcytometry	CD 133, CD 34	Not performed	(15)

*Cytokeratin (CK); Cluster of differentiation (CD); Smooth muscle actin (SMA); Stem *cells* antigen-1 (SCA-1); Octamer-binding transcription factor 4 (OCT4); sex determining region Y-box (SOX2); homeobox (NANOG); stage specific embryonic antigen (SSEA); Tumour resistance antigen (TRA)

DISCUSSION

Human breastmilk is a unique tailor-made body product that constantly evolved to satisfy infants and babies' nutritional and functional needs. It contains a special composition of nutrition, growth factors, bioactive and cellular components. Classically, the cellular part of breastmilk is mainly composed of breast-derived epithelial cells and blood-derived immune cells (22). Since the finding of potential stem cells in human breastmilk almost 15 years ago (12), many studies have been directed to discover the efficacy of human breastmilk in regenerative medicine. However, there is still a huge gap concerning a standardized method for the isolation of hBSCs.

Stem cells are undifferentiated cells with self-renewal capacity and can differentiate into different cell types (23). Pluripotent stem cells can be taken from ESCs or reprogrammed iPSCs, while mesenchymal stem cells can be taken from bone marrow and most of the connective tissue (3,4). The finding of hBSCs is promising because of the abundance source and its non-invasive technique.

The first question raised when deciding to use human breastmilk as a source of stem cells is whether the lactation stage affects the number of stem cells isolated. For decades, it has been known that breastmilk is a dynamic fluid produced by a mother up to several years after giving birth. The cellular composition and proportion of the cells within human breastmilk can be influenced by factors such as the stage of lactation, the processing method, or the nutritional and health status of mother and baby (24). This review study found that to isolate the hBSCs, the different lactation stages of breastfeeding can be used (Table.2). Although initially, the number of cells obtained from breastmilk is much more in colostrum than mature breastmilk, the hBSC is not much affected by the lactation stage. Some studies found that the percentages of hBSCs to be higher in mature milk (11,13), while another study reported that the marker of stem cells decreases after the 7th day of lactation (14).

The different results showed that there are probably other unexplained factors contributing to the population of stem cells in human breastmilk. The myriad of cellular components of breastmilk is evolved depend on the stage of lactation (25) or the requirement needed by the health status of mother and infant (26,27). Because there is no single article using either colostrum or mature breastmilk mentioned any difficulty in isolating the stem cells and that hBSCs taken from both stages can be differentiated into different lineages (Table 4), it can be said that the human breastmilk stem cells can be isolated from the various stage of lactation.

Currently, there is no standard protocol available for the isolation, culture and identification of this novel source of stem cells. The techniques used to extract the cell pellet were divided equally, with five articles centrifuging the breastmilk directly, five articles diluting with PBS, and five articles diluting with DMEM, demonstrating that there is no straining method for separating the cells in this study (Table 3). The washing times also range between one to three times between centrifuges. Only one article mentioned the difficulty in processing the cells due to the many cellular debris and lipids present.

Most of the articles seeding the cells in a culture, used the basal media DMEM as a medium, with some using the complex RPMI or balanced salt PBS. Various supplementation of growth factors implemented with or without the addition of antibiotics or antimycotic. The use of the best medium for cell culture is important as the medium acts as a feeder and works to instruct the cell fate (6). The additional mixture of nutrients, serum or growth factor, might be added to support the optimized microenvironment for stem cell maintenance. The optimized protocol of different SCs types keeps changing to answer the challenges of stem cells therapy (28), with the recent finding urge to replace the animal sera with the xeno-free platelet-rich plasma (PRP) to maintain SCs physiology (29). The study in the literature indicated that neither type of medium nor concentration of growth factor play a significant role in proliferating the hBSCs (14). Even without the addition of serum, the culture of human breastmilk can secret some of the growth factors such as Vascular endothelial growth factor (VEGF) and Hepatocyte Growth Factor (HGF) (9), both are essential in the proliferation, migration, and angiogenesis of stem cells (30).

All the studies harvested the cells in the early passage (up to passage 4) using Trypsin, Ethylene diamine tetra-acetic acid (EDTA), or manually harvested, which served as the three most common techniques for passaging (28). As summarized in Table 4, hBSCs expressed different stem cell markers in pluripotent and multipotent cell lines. But the contradiction of stem cells characterization in one or another study is somewhat confusing. The cells isolated from fresh breastmilk is expressing the typical MSC markers of CD90, CD105 and CD73 (7-11,14,17,18,20,21), typical ESC markers of OCT4, SOX2, and NANOG (12-14,18) or typical CD34+ of HSCs (7,11,14,15,18). However, a similar study conducted by another group is shown no evidence of the presence of MSCs (31), HSCs (20,21), or ESCs (18).

To be able to have a role in restorative medicine, the stem cells must be turned into specific cell types as needed to answer the question of modern therapy.

The hBSCs proved to be proficient at differentiating into all germinal layers that mimic the mammary cells, osteoblast, chondrocyte, adipocyte, hepatocyte, cardiomyocyte, and neurocytes in a specific medium (Table 4). Further study also revealed that hBSCs may possibly be involved in the development of tissues and organs in the infant. Studies in animals have proven that the cells pass the gastrointestinal tract mucosa and integrate into the organ of lactating pups (32) and rabbits (33). The studies on hBSCs are more interesting to overcome the future of cellular therapy, mostly because of easier access and flexibility in culture conditions. Nevertheless, different results in some study groups are probably due to different culture conditions used. Thus the optimal protocol is still needed to avoid bias.

CONCLUSION

Since finding stem/progenitor cells in human breast milk, several studies have used the hBSCs to further understand regenerative medicine. Unfortunately, the standard protocol has not been well explained. Our result showed that although the isolation and culture of hBSCs are somewhat more accessible compared to other sources of SCs, a standardized protocol is still needed. Breastmilk is a readily available body product that can be last up to 2 years or more. It can be used as a non-invasive alternative for stem cells sources as it is easy to obtain and contain various types of stem cells. But the result in characterizing hBSCs is still not clearly defined as one study contradicts the result of another study. Further experiments are still needed to standardize the protocol to avoid bias and confounding result.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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