METHOD OF PRODUCING MILK AND RELATED DAIRY PRODUCTS IN THE LAB USING STEM CELLS ISOLATED FROM DIFFERENT MAMMALS

FIELD OF THE INVENTION

The present invention provides a method to produce the entire composition of cow and other mammal's milk in the lab using stem cells or cell lines and recombinant methods or a combination of both.

BACKGROUND OF THE INVENTION

Milk is a dairy product; a nutrient-rich liquid food produced by mammalian mammary glands during or after pregnancy and is a primary source of nutrition for infant mammals including humans. It is considered a balanced diet due to its unique beneficial properties that are attributed to its composition. Generally it is composed of lipids (mono-, di- and triglycerides, phospholipids, free fatty acids, cholesterol and cholesteryl esters), proteins (casein and whey proteins), vitamins (vitamin A, B1, B2, B5, B6, B12, C, D, E and K), minerals (calcium, potassium, magnesium, phosphate, sodium, chloride, citrates and calcium-phosphate), carbohydrates and sugars (glucose, lactose, galactose, and other oligosaccharides). However, the exact composition of milk varies from species to species. The pH of milk varies from 6.4 – 6.8. Other components of milk consist of stem cells, mammary gland cells, white blood cells, enzymes, bacteria, and immunoglobulins. Besides being rich in nutrients, milk is a source of various dairy products such as yogurt, cream, cheese, butter, and ice cream.

In 2017, 278 million dairy cows were predicted in the world that produced 828 million tons (72.5 billion liters) of milk. With the annual average global per capita consumption of 108-liter milk, milk consumption is estimated to be 81 billion liters. Whereas the number of cows has declined (0.2%) since 2016 and with the increasing population of the world, milk consumption is predicted to rise. The global dairy market volume is estimated to be 216 metric tons (of which 54% market share is taken up by liquid milk) with the global annual sale of 674 billion USD and it is projected to increase (Current Worldwide Total Milk Consumption per capita, http://chartsbin.com/view/1491; Milk Production, http://www.fao.org/dairy-production-products/production/en/; World Cow Numbers,

https://dairy.ahdb.org.uk/market-information/farming-data/cow-numbers/world-cow-numbers/; Statista Research Department, www.statista.com).

Though milk is a healthy diet, the trend of replacing milk with its alternatives is increasing due to concerns like lactose intolerance, ingestion of steroids and other hormones with the milk, ethical concerns and decline of dairy cattle. The alternative sources of milk include plant-based milk such as soy, oats, almonds, cashew nuts, coconut, flaxseed, rice and tiger nut milk and milk produced by recombinant or chemical synthesis methods. The consumption of present milk substitutes is disadvantageous as they lack one or more components of dairy milk, thereby compromising their flavor and nutritional value. This urges the search of protocols and methods to produce milk with the same or superior composition of milk and related dairy products.

BRIEF SUMMARY OF THE INVENTION

The invention is a method to produce milk using stem cells, cell lines, and recombinant methods or their combination to create a product with similar or better properties compared to dairy milk by isolating and differentiating the cells from mammalian source or using commercial cell lines, differentiate them into mammary epithelial and luminal cells and induce them to lactate in 2D and 3D culture systems.

In one embodiment of the invention, where the application is the isolation of cells from mammalian sources, the cells are stem cells isolated from adipose tissue, milk, bone marrow or umbilical cord and source is humans and species of cow, buffalo, camel, goat, and sheep.

In one embodiment of the invention, where the application is to grow, expand and differentiate the cells, the growth medium used is RPMI-1640, α-MEM, DMEM or DMEM/F12 containing 10% FBS, antibiotics (optional) and glutamine, the cells are grown initially in 2D culture and later transferred to 3D culture system to differentiate along with 2D differentiation. The differentiation medium is free of fetal bovine serum and contains inducers for lactation such as insulin, prolactin, hydrocortisone, etc.

In one embodiment of the invention, where the expression of marker genes of differentiation and lactation is analyzed, real-time PCR, western blot analysis, and flow cytometry of cell

surface marker for mammary epithelial and mammary luminal cells such as CK14 and CK18; and the same of lactation markers such as casein, lactoglobulin, α-lactalbumin, lactoferrin, leptin, and butrophilin are employed.

In one embodiment of the invention, where milk secreted is separated from the culture medium, microfiltration/ ultrafiltration is employed to remove media components from milk. The milk concentrate and separated whey proteins are mixed later or used to form dairy products such as cheese, yogurt, butter, lactose-free, low fat or low/ high protein milk.

In one embodiment of the invention, the composition of growth medium is customized in a way that ensures cell growth and is not needed to be removed from the milk. The inducers added are removed by flow cytometry or denatured by pasteurization.

In one embodiment of the invention, where the fatty acid content in the milk is analyzed, GCMS, NIRS, NMR, and RS is performed to detect fatty acids in the milk.

In one embodiment of the invention, where vitamins are detected in milk, LCMS, HPLC with immune-affinity columns and ELISA based assay kits are used for vitamin detection.

In one embodiment of the invention, where minerals and trace elements are detected in milk, colorimetric assays and atomic absorption spectroscopy for detection of minerals, and flameless atomic absorption spectroscopy for detection of trace elements is employed.

In one embodiment of the invention, where the composition of milk is analyzed, the composition of milk and any remaining component is detected by the milk analyzer.

DETAILED DESCRIPTION OF THE INVENTION

The product provided herein is a dairy product produced by mammalian cell culture, with composition comparable to natural milk. The invention is based on the fact that cells can be isolated and grown in the lab and induced to lactate. Furthermore, the invention is based on the fact that the cells upon induction secrete milk proteins and other components in the medium that can be separated from the culture media and processed.

In one embodiment, the cells are stem cells isolated from adipose tissue, umbilical cord, umbilical cord blood, mammary gland and milk of cow, buffalo, goat, sheep, camels, and humans. In other embodiments, the isolated cells are grown and cultured in the lab in 2D and 3D culture systems. The cells are differentiated into mammary epithelial and mammary luminal cells and induced to lactate. The cells upon induction secrete milk components in the medium that can be isolated and processed as milk. The composition of milk produced is comparable to natural milk.

In the first embodiment, stem cells are isolated from adipose tissue of different aforementioned mammals. Fresh adipose tissue was obtained from slaughterhouses or liposuction (in case of humans, after informed consent), kept in phosphate buffer saline supplemented with antibiotics and transferred to the lab. In the lab, enzymatic digestion of adipose tissue was carried out followed by centrifugation to isolate mesenchymal stem cells. The mesenchymal stem cells were transferred to cell culture flasks and grown under standard growth conditions i.e. 37 ^{o}C, 5% CO_2 (Ahmad and Shakoori. 2013. *J Stem Cell Regen Med.* 9(2): 29- 36). DMEM-F12, RPMI, and Alpha-MEM medium supplemented with 15% fetal bovine serum, 1% antibiotics were used for initial culture and were replaced with 10% fetal bovine serum-supplemented media after the first passage.

One example of the first embodiment is the isolation of mesenchymal stem cells from the umbilical cord/ umbilical cord blood of mammals. The umbilical cords and cord blood of the aforementioned animals with healthy pregnancies were obtained at the end of the gestation period from the veterinary hospital immediately after delivery. For humans, the umbilical cord and cord blood of healthy pregnancies were obtained at the end of the gestation period from the hospital after maternal consent to donate cord and cord blood for the project. The umbilical cord was collected in phosphate buffer saline supplemented with antibiotics and cord blood was collected in tubes with anticoagulant and transferred to the lab. The umbilical cord was cut into 5 cm^2 pieces under sterile conditions, blood vessels were removed and the segments of umbilical cord were transferred in cell culture plates in DMEM-F12, RPMI, and Alpha-MEM supplemented with 10% fetal bovine serum, and 1% antibiotics for seven days. Explants were removed and media was changed after seven days with fresh growth medium. Mesenchymal stem cells from cord blood were isolated by density gradient centrifugation by

adding Ficoll-Paque solution, cells from ficoll-media interphase were collected and added to the aforementioned complete media. Growth medium was supplemented with dexamethasone to reduce adherence of monocytes. Non-adherent cells were removed after seven days of isolation by changing the culture medium with fresh complete growth medium (Tropel et al. 2004. *Exp Cell Res.* 295(2): 395-406; Van Pham et al. 2016. *Cell Tissue Bank*. 17(2): 289 - 302; Hassan et al. 2017. *Int J Stem Cell*. 10(2): 184-192).

Another example of the first embodiment is the isolation of mesenchymal stem cells from healthy mammary tissue of the aforementioned lactating mammals. Isolation is not done from human breast tissue due to ethical issues. All other animals mentioned are utilized for this type of isolation. Fresh breast tissue is obtained from slaughterhouses and is washed with phosphate buffer saline (PBS). The tissue is cut in 1 mm^3 pieces and kept in the complete culture medium under standard culture conditions. Alternatively the tissue was subjected to enzymatic digestion followed by density gradient centrifugation. The cells were washed thrice with phosphate buffer saline and seeded in culture plates in DMEM-F12, RPMI, and Alpha-MEM medium under standard culture conditions. After preliminary plating, the media is changed every two or three days according to requirement. Adherent fibroblast-like cells appear after ten days of culturing. These cells are trypsinized into another flask for expansion. The cells of passage 3 are further used for differentiation into mature breast luminal and epithelial cells (Huynh et al. 1991. *Exp Cell Re*s. 197(2): 191 -199; Gibson et al. 1991. *In Vitro Cell Dev Biol Anim.* 27(7): 585-594; Blatchford et al. 1999. In *Animal Cell Technology: Basic & Applied Aspects*. Springer, Dordrecht. 141-145; Zhang et al. 2013. *Oncol. Lett.* 6(6): 1577-1582).

Another example of the first embodiment is the isolation of mesenchymal stem cells from milk, for which fresh milk was collected from the aforementioned mammals under aseptic conditions. An equal volume of phosphate buffer saline was added to diluted milk, followed by centrifugation for 20 min. The cell pellet was washed thrice with phosphate buffer saline and cells were seeded in cell culture flasks in DMEM-F12, RPMI, and Alpha-MEM medium supplemented with 10% fetal bovine serum and 1% antibiotics under standard culture conditions (Hassiotou et al. 2012. *Stem Cells*. 30(10): 2164-2174).

In the second embodiment of the invention, the cells isolated from various sources were differentiated in mammary epithelial and mammary luminal cells in 2D and 3D culture systems. For 2D culture, the cells were initially seeded in culture plates in growth media supplemented with 10ng/ml epithelial growth factor and 5µg/ml insulin. At confluence, cells were fed with growth medium supplemented with 2% fetal bovine serum, 1% antibiotics, and 5µg/ml insulin for 48h. To induce differentiation, the cells were fed with complete growth medium containing 5µg/ml insulin, 1µg/ml hydrocortisone, 0.65ng/ml triiodothyronine, 100nM dexamethasone, and 1µg/ml prolactin. After 24h, antibiotics, and serum were removed from the complete induction medium. For 3D culture, the cells were trypsinized and cultured in matrigel, hyaluronic acid, or ultra-low attachment surface culture plates for six days and induced to differentiate and lactate by adding the aforementioned differentiation and lactation medium (Huynh et al. 1991. *Exp Cell Re*s. 197(2): 191 -199; Gibson et al. 1991. *In Vitro Cell Dev Biol Anim.* 27(7): 585-594; Blatchford et al. 1999. In *Animal Cell Technology: Basic & Applied Aspects*. Springer, Dordrecht. 141-145; Williams et al. 2009. *Breast Cancer Res*. 11(3): 26-43; Arevalo et al. 2015. *Am J Physiol Cell Physiol.* 310(5): C348 - C356).

CLAIMS

[1] Methods for isolation of stem cells from various mammalian sources. Step a. Mesenchymal stem cells are isolated from various adipose tissues, umbilical cord cells, umbilical cord blood, healthy mammary tissues of lactating mammals and fresh milk of various mammalian sources. Step b. These cells are provided with a suitable medium for growth in laboratory culture flasks.

[2] The above isolated and cultured mammalian stem cells are allowed to differentiate in 2D and 3D culture systems. Differentiated cells are then induced to produce milk having a composition similar to mammalian milk.