Human Colostrum is a Rich Source of Cells with Stem Cell-Like Properties

Sam Vijay Kumar J, Harikrishnan Rajkumar, Silambarasi Nagasamy, Seethesh Ghose, Balanehru Subramanian, Adithan C

ABSTRACT

Background and Objective: Stem cells have been isolated from various parts of the human body including bone marrow, umbilical cord blood and adipose tissue. Human breast milk as a source of stem cells is less studied with limited reports. In this pilot work, we have investigated the stem cell-like properties of cells derived from human colostrum using in vitro mechanistic assays.

Material and Methods: Institutional Committee for Stem Cell Research's approval and written informed consent from volunteering mothers were obtained prior to sample collection. Colostrum (n=4) samples were collected from healthy lactating mothers of neonates admitted in neonatal intensive care unit and postnatal wards. Cells from colostrum were cultured in Minimal Essential Medium – Alpha containing 10% Fetal Bovine Serum. These adherent growing cells were propagated in vitro and investigated for their differentiation potential into adipogenic and osteogenic lineages, anchorage independent spherogenesis and clonality under mammalian cell culture conditions.

Results: Human colostrum was found to be richly cellular. Colostrum derived cells were capable of bi-lineage differentiation into osteogenic and adipogenic lineages within 7 days of induction. Colostrum derived cultures exhibited clonal growth and expansion under in vitro cell culture conditions. Colostrum derived cells also showed anchorage independent growth as spheres.

Conclusion: This study provides compelling and pioneering evidence to cite that human colostrum is a rich source of stem cell-like cells. The study took into account in vitro functional properties of adherence, clonal expansion and bi-lineage differentiation. Validation with a larger cohort and multiple profiling of cells would further open newer vistas aimed at furthering the present understanding and value of colostrum stem cells in maternal-perinatal physiology.

Keywords: Colostrum, Breast milk, Stem Cells, Differentiation

INTRODUCTION

Stem cells are undifferentiated, with characteristic hallmark features of self-renewal, proliferation and differentiation. They share important functions in the body including tissue homeostasis, regeneration and repair. Stem cells have been isolated from various organs and tissues of the human body including blood, liver, bone marrow, gut, and brain. While human breast milk is among the lesser studied sources for stem cells, colostrum, which is biologically different and secreted during the initial days post parturition, has not been explored yet for its full potentials to be a source of stem cells.

Reports of cells with multi-lineage differentiation potential in human breast milk have caused a huge interest in the field of lactation and perinatal health. Thus the potential of breast milk as a source of stem cells is evoking increased interest. There is limited evidence for the existence of cells of diverse lineages in human breast milk, such as haematopoietic, mesenchymal and neuro-epithelial lineages. Human breast milk has also been reported to contain cells expressing
markers of pluripotency such as the transcription factors OCT-4, NANOG and SOX2. Human milk has also been shown to contain colony stimulating factor, known to help in the proliferation and maintenance of stem cells. Nevertheless, enumeration of these stem cells and their role in lactation is still in its infancy.

Colostrum, secreted in the first few days after parturition, is biologically distinct from breast milk. The nutritional and immunological significance of human colostrum in post-natal well-being is well documented. Colostrum and its potential for stem cells have not been explored. But for a report on bovine colostrum containing osteogenic factors, there is no information on colostrum and its relevance to stem cells and regeneration.

Reports of isolation of cells with stem cell characteristics from breast milk are beginning to accumulate. But exploration of stem cell characteristics from human milk is still lagging with a vast majority of the existing reports being performed on animal based models and tissue explant cultures. Exploration of colostrum which is distinct from breast milk has not been reported yet. Thus there is a decisive need to explore, isolate and characterize stem cells from human colostrum. The present study is aimed at investigating colostrum derived cells with reference to properties of multi-lineage differentiation potential and clonality.

**MATERIAL AND METHODS**

**Sample preparation**

This study was approved by the Institutional Committee for Stem Cell Research (IC-SCR) of Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth, Puducherry, India. All the participants were explained about the procedure and written informed consent was obtained prior to sample collection. Colostrum samples were collected from healthy lactating mothers (aged between 20 to 30 yrs.) of neonates admitted in the neonatal intensive care unit (NICU) and postnatal ward of our hospital. Breast and nipple were wiped with sterile water and also with 70% ethanol prior to sample collection. Manually expressed colostrum (5-10ml) was collected from each participant in a sterile container and immediately transported in a thermally insulated container containing ice to the Central Inter-Disciplinary Research Facility for further processing. Four colostrum samples were collected. The samples were processed within 30 minutes of collection.

**Mammalian cell culture**

A modified mammalian cell culture protocol for isolating cells from colostrum samples was adapted from Hassiotou et al. Briefly, the samples were diluted with equal volume of Dulbecco’s Modified Eagle’s Medium (DMEM) containing 1% Fetal Bovine Serum (FBS). The samples were centrifuged at 800g for 20 minutes at 4 degrees Celsius. The supernatant was discarded and the cell pellet was washed twice by suspending in DMEM containing 1% FBS and spinning at the same speed as above. The final cell pellet was suspended in DMEM, 10% FBS, 100units/ml penicillin and 100µg/ml streptomycin. Viability of the cells was checked with 0.4% Trypan blue dye exclusion test. The cells were seeded into 25 cm² culture flasks at a concentration of 5×10⁶ to 5×10⁷ per flask. The flasks were incubated at 37°C and 5%CO₂ in a water jacketed incubator. Non-adherent cells were removed after 7 days. Thereafter the culture medium was refreshed every 3-4 days. The adherent cells in culture were maintained for 3 weeks prior to assessing their differentiation capability.

**Differentiation**

Cells that were expanded for 3 weeks were used for the differentiation experiments.

Adipogenic induction protocol was adapted from Janderová et al. The adipogenic induction medium consisted of Dulbecco's Modified Eagle's Medium (DMEM), 4.5 g/L glucose, 1µM dexamethasone, 0.2mM indomethacin, 10µg/ml insulin, 0.5mM 3-isobutyl-1-methylxanthine, 10% fetal bovine serum, 100 units/ml penicillin and 100µg/ml streptomycin. Cells were treated with the induction medium for 7 days. Adipogenesis was verified by Oil O Red (0.1%) staining.

Osteogenic induction protocol was adapted from Jaiswal et al. The osteogenic induction medium consisted of Minimal Essential Medium – Alpha (MEM-α),100nM dexamethasone, 10mM beta-glycerophosphate, 0.05mM ascorbic acid, 10% fetal bovine serum, 100 units/ml penicillin and 100µg/ml streptomycin. Cells were treated with the induction medium for 7 days. Osteogenesis was verified by Alizarin Red (2%) staining.

Stained cells were visualized under an inverted phase contrast microscope (Zeiss Axiosvert 40 CFL) and pictures were taken using Zeiss Axioscam ER 5s colour camera and ProgRes CapturePro v2.8.8 software.
Anchorage independent growth

Fresh colostrum derived cells were seeded on to culture dishes coated with Poly-HEMA, which would help in forced suspension of the cells. The cells were viewed at the end of 4 days for the presence of clones of cells exhibiting the capability of survival in anchorage deprived conditions, a hallmark of cancer cells.

RESULTS

Cell culture

Colostrum (n=4) samples were processed within 30 minutes of collection. There was a rich concentration of cells in colostrum (1.64 millions/ml). More than 99% cells were viable in all the samples (Table 1). No marked difference in the viability of cells between the different samples demonstrated consistency in sample handling protocols from collection to culture. Plastic adherent fusiform cells were observed in all the samples at the end of 3 days. From the perspective of morphology, diversity of cells in colostrum derived cultures were observed; spindly to more rounded cells. By 10 days all the colostrum derived cultures started depicting clonal growth as evidenced by distinct tightly packed colonies of cells (Figure 1) which expanded upon culture (Figure 2).

Differentiation and anchorage independent growth

Four different experiments were performed using colostrum derived cells to assess their differentiation capability – into adipogenic and osteogenic lineages. Adipogenic and osteogenic differentiation were observed in all colostrum derived cell cultures at the end of 7 days of induction. These were confirmed by positivity in Oil O Red and Alizarin Red staining indicating fat globule formation and calcium mineralization respectively in the cultures (Figure 3). Anchorage independent growth which is also a characterized property of stem cells and cancer cells was demonstrated in colostrum derived cells. All cultures yielded spherogenic growth on poly-HEMA coated plates. (Figure 4)

Table 1: Characteristics of Human colostrum samples

<table>
<thead>
<tr>
<th>Expt.No</th>
<th>Volume of Sample (ml)</th>
<th>Viability of cells (%)</th>
<th>Total Cell Count (million)</th>
<th>in vitro growth</th>
<th>in vitro clonal growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUC-01</td>
<td>10</td>
<td>99</td>
<td>3.6</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HUC-02</td>
<td>5</td>
<td>99</td>
<td>19.2</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HUC-03</td>
<td>5</td>
<td>99</td>
<td>8.1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HUC-04</td>
<td>5</td>
<td>99</td>
<td>10.1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Figure 1: Clones of cells in colostrum derived cultures (10x magnification)

Figure 2: Expanding clone in colostrum post 14 days culture (10x magnification)
DISCUSSION

Here, we report for the first time, the successful harvest and maintenance of cells from human colostrum under *in vitro* mammalian cell culture conditions. This is also the first report attempting to assess the cellular properties of differentiation capability and clonal growth in these cells.

Maternal-perinatal cellular transfer has been reported few decades ago using a rat model. Nevertheless, there is no study to the best of our knowledge on human colostrum derived cells in infant gut development. However a corollary described by Miller et al using a porcine explant culture model supports trans-epithelial trafficking of maternal colostral leucocytes cells in neonatal pigs. Another report by Hassiotou et al using a murine model demonstrated cellular transfer from milk to stomach wall and thymus. Engraftment of rabbit milk derived MSC into the organs of the offsprings has also been reported recently. Our observations of rich cellular content in colostrum along with *in vitro* growth of these cells indicate their plausible role in cellular trafficking and development of intestine in newborns.

Clonal expansion and extensive proliferation *in vitro* are some of the cardinal features of different types of stem cells including mesenchymal stem cells, neural stem cells and intestinal stem cells. Our experience of clonal cells *in vitro* from human colostrum derived cultures demonstrates the existence of cells with self-renewal property. The property of these clones to expand upon extended culture is another hallmark property of stem cells – proliferation. This suggests the existence of cells with stem cell-like property in human colostrum. Our data along with above mentioned reports definitely hint towards possible role and involvement of colostrum derived cells in the development of infant gut, which has to be verified.

Evidence exists for breast milk as a source of multi-lineage cells including mesenchymal stem cells and neurogenic cells. However, report of adipogenic and osteogenic cells *in vitro* in colostrum derived cells has not been reported hitherto. Our observation of such bi-lineage differentiating cells in human colostrum applied along with the limited corpus of literature on breast milk derived osteoinductive factors, colony stimulating factors and differentiating cells only furthers our belief that colostrum is more than just a nutritional source – it is a natural source of stem cells for the new born.
The facts drawn from our present study strengthens our belief that colostrum contains cells that are more amenable to differentiation under a short induction period along with clonality. A major proportion of immune network in a developed individual exists in the Gut Associated Lymphoid Tissue (GALT) and newborns have an underdeveloped immune system. With the above discussed results it only seems reasonable to propose that colostrum derived multi-lineage cells might play an important role in the establishment of GALT and other aspects of neonatal physiology. The cellular diversity of colostrum suggests likely interaction and interplay of the cellular and non-cellular factors present in colostrum, which could facilitate the establishment of a newborn's digestive, nervous and skeletal systems. However validation of these results with a larger cohort is imperative and strongly mandated. The limitations of this study are the lack of molecular markers for stemness, absence of breast milk derived data and higher sample numbers; We propose to address them objectively in our future studies.

This is one among the few reports of the presence of cells with adipogenic and osteogenic potential in human colostrum. We put forth the premise that colostrum is a rich source of cells with stemness properties. This study gives preliminary evidence in support of colostrum as a rich source of cells with stem cell-like property. We intend to employ a multi-dimensional profiling approach with more molecular and functional stem cell markers to unravel more on the characteristics of colostrum derived cells and their clinical significance. It is imperative that a larger study to promulgate the validation of human colostrum as a rich source of stem cells is deemed necessary, besides enhancing our comprehension of the biological significance of these cells along with breast milk derived cells in neonatal health and well being.

CONCLUSION

This study provides evidence for colostrum as a source of stem cell-like cells by using in vitro properties of adherence, clonal expansion, anchorage independent sphere formation and bi-lineage differentiation. The presence of stem cell-like cells with clonality and bi-lineage differentiation potential in colostrum designates it is a rich source of stem cell-like cells for the new born. The relevance of these cells in maternal-perinatal biology has to be investigated and corroborated with a larger cohort of participants, albeit our results along with presently available literature suggest plausible role of colostrum derived stem cell-like cells in the development of the newborn’s intestine.

ABBREVIATIONS

OCT-4, octamer-binding transcription factor 4; NANOG, Nanog Homeobox; SOX2, SRY (sex determining region Y)-box 2; NICU (Neonatal intensive care unit); DMEM, Dulbecco’s Modified Eagle’s Medium; FBS, Foetal Bovine Serum; MEM-α, Minimal Essential Medium-Alpha Modification; GALT, Gut Associated Lymphoid Tissue

CONFLICT OF INTEREST

None.

FUNDING

This work has been supported partly through the Indian Council of Medical Research-Short Term Studentship (ICMR-STS). The authors acknowledge the financial support by the Management of Sri Balaji Vidyapeeth.

ACKNOWLEDGEMENT

The authors wish to acknowledge the kind support of Prof. G.Subramanian, Advisor, Central Inter-Disciplinary Research Facility, Pondicherry.

REFERENCES

9. Thomas E, Zeps N, Cregan M, Hartmann P, Martin T. 14-3-3-sigma (sigma) regulates proliferation and differentiation of multipotent

26
An article in Lancet, reported the findings of a large, epidemiological cohort study of individuals aged 35–70 years in 18 countries with a median follow-up of 7•4 years (IQR 5•3–9•3). Dietary intake of 135 335 individuals was recorded using validated food frequency questionnaires. The primary outcomes were total mortality and major cardiovascular events (fatal cardiovascular disease, non-fatal myocardial infarction, stroke, and heart failure). Secondary outcomes were all myocardial infarctions, stroke, cardiovascular disease mortality, and non-cardiovascular disease mortality. Participants were categorised into quintiles of nutrient intake (carbohydrate, fats, and protein) based on percentage of energy provided by nutrients. The associations between consumption of carbohydrate, total fat, and each type of fat with cardiovascular disease and total mortality were assessed. Hazard ratios (HRs) was calculated using a multivariable Cox frailty model with random intercepts to account for centre clustering.

Findings: During follow-up, we documented 5796 deaths and 4784 major cardiovascular disease events. Higher carbohydrate intake was associated with an increased risk of total mortality (highest [quintile 5] Vs lowest quintile [quintile 1] category, HR 1•28 [95% CI 1•12–1•46], ptrend=0•0001) but not with the risk of cardiovascular disease or cardiovascular disease mortality. Intake of total fat and each type of fat was associated with lower risk of total mortality (quintile 5 vs quintile 1, total fat: HR 0•77 [95% CI 0•67–0•87], ptrend<0•0001; saturated fat, HR 0•86 [0•76–0•99], ptrend=0•0088; monounsaturated fat: HR 0•81 [0•71–0•92], ptrend=0•0001; and polyunsaturated fat: HR 0•86 [0•71–0•89], ptrend=0•0001). Higher saturated fat intake was associated with lower risk of stroke (quintile 5 vs quintile 1, HR 0•79 [95% CI 0•64–0•98], ptrend=0•0498). Total fat and saturated and unsaturated fats were not significantly associated with risk of myocardial infarction or cardiovascular disease mortality.

Interpretation: High carbohydrate intake was associated with higher risk of total mortality, whereas total fat and individual types of fat were related to lower total mortality. Total fat and types of fat were not associated with fatal cardiovascular disease, non-fatal myocardial infarction, or cardiovascular disease mortality, whereas saturated fat had an inverse association with stroke. Global dietary guidelines should be reconsidered in light of these findings.

Source: Lancet, 29 August 2017, DOI: http://dx.doi.org/10.1016/S0140-6736(17)32252-3