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Original Article

Comparison of Colony-forming Efficiency between Breast Milk Stem/progenitor Cells of Mothers with Preterm and Full-term Delivery

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ABSTRACT

Background: The beneficial effect of breastfeeding for the health of mothers and infants are well recognized. Breast milk is a novel source of the stem cells forming during pregnancy and lactation. In the present study, the colony forming efficiency (CFE) of breast milk-derived stem/progenitor cells in the breast milk of mothers with preterm delivery (gestational age of fewer than 37 weeks) was compared with that of mothers with full-term delivery (gestational age of more than 37 weeks).

Methods: Fresh-pumped breast milk of 30 healthy mothers with full-term delivery and 30 mothers with preterm delivery who had no underlying illness and drug intake were collected on days 5 and 15 after delivery, and then immediately assessed. For the purpose of the study, 10 ml fresh breast milk was gently mixed with equal amount of phosphate buffer saline, centrifuged at 1,380 rpm for 20 min, cultivated in 1 ml MethoCult H4435 medium (Stem Cell Technologies), and incubated at 37°C with 5% CO_2 and 80% humidity for 14 days.

Results: The CFE is significantly lower in the 25 to 35-year-old mothers on day 15 after delivery than in 35 to 45-year-old mothers (P=0.01). In both groups of mothers, the CFE was higher on day 5 than on day 15. Moreover, a significant correlation was observed between the CFE of breast milk stem/progenitor cells obtained on days 5 and 15 with the infants weighing 3,000-4,000 g (130±62, P=0.03 and 105±26, P=0.021), respectively. Furthermore, CFE increased in the breast milk of mothers aged 35 $\geq$ years in comparison to that of younger mothers.

Conclusion: According to our analysis, breast milk stem/progenitor cells CFE was higher in mothers with preterm delivery than in mothers with full-term delivery. These observations may uncover the compensatory mechanisms illustrated in the mothers' breast milk to improve the preterm infants' tissues development and organ formation in which various factors were involved, such as mothers' age and infants' weight.

Keywords: Breast milk stem/progenitor cells, Colony forming efficiency, Full-term delivery, Preterm delivery

Introduction

The human breast milk is a complicated biological fluid, characterized as a unique nutritional resource for both term and preterm neonates (1). The infant who is born with 38-42 weeks of gestation is described as a term infant, while a preterm infant is born before completing 37 weeks of gestation (2). The rates of mortality and morbidity are higher in preterm infants who are born immaturely, so they need special protection, compared to mature full-term infants (3). Based on the World Health Organization (WHO) annual birth report, it has been estimated that 15 million preterm infants are born every year, and this amount is rising. Breast milk protects offspring against respiratory and gastrointestinal diseases, particularly in preterm infants, and it has been taught that it improves cognitive performance, organ formation, and

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tissue development (4, 5).

The breast milk composition in mothers with preterm infants is fundamentally different in comparison with that in mothers with full-term infant delivery (6). Several studies represent that breast milk has a unique species-specific composition (7) influenced by infant's sickness, organ infection, environmental elements, and genetic factors, as well as maternal diet and mental condition (6, 8-13). The existence of lactoferrin, secretory immunoglobulin A (sIgA), and lysozyme in the breast milk of mothers with preterm delivery demonstrates the protective effect of this substance against infection (14). Furthermore, the distinct advantages of long-chain free fatty acids include arachidonic (20:4n-6) and docosahexaenoic (22:6n-3) acids relating to vision and cognition (15). In addition, oligosaccharides protect the mucosal layer against bacterial attachments and prevent a consequent systemic infection; therefore, they are the benefits which may impact the long-term health and development.

Recent breakthroughs have illustrated that breast milk stem/progenitor cells are involved in infant's tissue development and regeneration (9). It has been published that the breast milk of mothers with preterm infant provides different macro- and micronutrients in comparison to that of mothers with term infants (6, 16). According to the involvement of breast milk stem/progenitor cells in infant's growth and tissue development, and other variable aspects of breast milk composition, the difference between breast milk stem cell characteristics of mothers with preterm and term infants may be considering.

It is thought that after ingestion, breast milk stem cells circulate the neonate's body and promote the organ development and tissue homeostasis (17). In the current study, we compared the colony forming efficiency (CFE) of breast milk stem/progenitor cells between mothers with preterm infants and those with full-term infants.

Methods

Participants and sample collection

Based on the gestational week, two groups of mothers with no underlying illness or drug intake were enrolled in our pilot study. Our study was conducted on 30 mothers with preterm delivery (gestational age of < 37 weeks) and 30 mothers with full-term delivery (gestational age of 40±2 weeks). Due to the pilot nature of the study and the low parental involvement, a limited number of participants were selected.

The mothers with normal full-term delivery were recruited from the Maternity Ward of Imam Reza Hospital, Mashhad, Iran, whereas the mothers with preterm delivery were recruited from the Neonatal Intensive Care Unit (NICU) of the same hospital. Our inclusion criteria were left purposefully with the large inclusion range even though the enrolled infants ended up similarly in gestation (Table 1). The infants were categorized based on gender, height, weight, and head circumference according to the WHO and the national center for health statistics standard charts (18).

The infants were fed only with their mothers' breast milk. The complete explanation of the study was given to each participant, and informed consent was provided by all mothers. For the purpose of the study, 10 ml fresh pomp-expressed breast milk was collected from each participant on days 5 and 15 after delivery and immediately assessed.

Cell isolation

An equal volume of sterile phosphate buffered saline (PBS) (pH=7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) was added to each sample and centrifuged at 1,340 rpm for 20 min at 4°C. The upper high-fat layer and liquid part were completely removed, and the cell pellet was washed three times with sterile PBS. After third centrifugation, the cell pellet was resuspended in 1 ml of the previously removed liquid part containing 15% fetal bovine serum (FBS), and consequently cultivated in 1 ml MethoCult H4435 medium (Stem Cell Technologies) in order to determine the CFE. The cells were counted with

Table 1. Maternal age, gestational age, and neonatal height, gender, and head circumference

Case	Variable	Group	Frequency
Mothers	Age	<35 years	26
		≥35years	34
	Gestational age	<37 week	30
		40±2 week	30
Infants	Height	35-45 cm	29
		45-60 cm	31
	Sex	Male	31
		Female	29
	Weight	1000-2000 gr	22
		2000-3000 gr	21
		3000-4000 gr	17
	Head circumference	20-25 cm	6
		25-30 cm	13
		30-35 cm	25
		35-40 cm	16

the Neubauer hemocytometer slide, and the cellular viability of each sample was determined by trypan blue exclusion.

Cell culture

In order to evaluate the CFE of human breast milk stem/progenitor cells, 10^7 cells, obtained from the previous step, were seeded in 1 ml of media (MethoCult H 4435, Stem cell Technology) and cultivated in the central wells of 24-well plates. The samples were cultured in a duplicate manner and incubated at 37°C with 85% humidity and 5% CO_2 for 2 weeks. After the incubation time, all of the vital colonies were calculated by an inverted microscope.

Statistical analysis

Based on the obtained data, different statistical tests were applied. The Kolmogorov-Smirnov test was performed for the determination of the normality of the data. The Student's t-test and independent t-test were performed for the comparisons of two related and two unrelated groups. The one-way ANOVA with the Scheffe's post-hoc test was used to compare the variables between the two groups. The SPSS (version 20, IBM SPSS Statistics) and GraphPad Prism (version 3, San Diego, CA) were employed for data analysis. All statistical tests were performed two-tailed to estimate the p-value which was considered to be statistically significant at < 0.05.

Results

The preterm group comprised 19 (63%) males and 11 (36.7%) females, while the full-term group consisted of 12 (40%) and 18 (60%) males and female, respectively. Furthermore, the results illustrated that in the preterm and full-term groups, 14 and 1 infants were born through cesarean section, while 16 and 29 cases were delivered normally, respectively. The obtained data demonstrated that the mean weight, height, and head circumference in the preterm infants were 2262.5±1542 g, 44.5±9.3 cm, and 29±9 cm, whereas in the full-term infants, these values were 2465±1015 g, 43±11.5 cm, and 29.75±7.25 cm, respectively.

Interestingly, our data demonstrated the significant increase in breast milk CFE of mothers with infants' weight of 3,000-4,000 g on day 5 (130±62; P=0.03) and day 15 (105±26; P=0.021) after delivery. Furthermore, the CFE on day 15 after delivery in the 25 to 35-year-old mothers was significantly lower than that in 35 to 45-year-old mothers (P=0.01).

Figure 1. Appearances of the breast milk stem/progenitor cells 14 days after incubation in a 12-well plate with an 18-mm diameter and containing 1000 µl Methocult H 4435 medium (The numbers of colonies were assigned with inverted microscope [original magnification 40×])

In the breast milk of mothers with full-term delivery and preterm delivery, the CFE on day 5 was higher than that on day 15. Moreover, a significant correlation was observed among breast milk stem/progenitor cells CFE on days 5 and 15 and the infants weighing 3,000-4,000 g (130±62, P=0.03 and 105±26, P=0.021; Figure 1) respectively. Furthermore, CFE increased in the breast milk of mothers aged 35 ≥ years in comparison to that of younger mothers. Compare with the elder mothers, breast milk CFE increased significantly on days 5 and 15 after delivery in younger mothers (P<0.05) which shows the potential of breast milk CFE in younger mothers. As well as, our results revealed that the breast milk CFE on day 5 was marginally lower in 25 to 35-year-old mothers in comparison to that in the 35 to 45-year-old mothers (P=0.082; Figure 2).

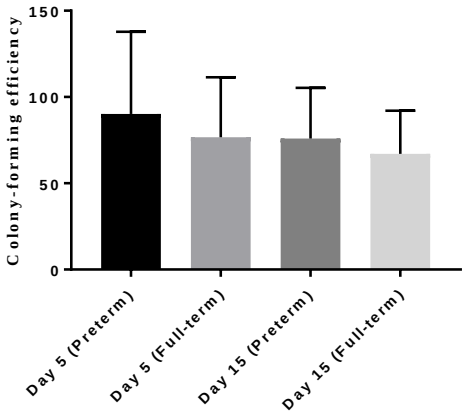

Figure 2. Comparison of colony forming efficiency between mothers' breast milk with preterm and full-term delivery

Table 2. Correlations of colony forming efficiency in the breast milk of mothers with preterm and full-term delivery on days 5 and 15

Gestational age	Sample	Frequency	Pearson correlation
Preterm < 37 weeks	Day 5	30	0.648**
	Day 15	30	0.648**
Full-term 40±2 weeks	Day 5	30	0.734**
	Day 15	30	0.734**

**. Correlation is significant at the 0.01 level (2-tailed).

Table 3. Comparison of the colony-forming efficiency of breast milk stem/progenitor cells in mothers with preterm and full-term delivery on days 5 and 15 after delivery

Gestational age	Sample	Frequency	Minimum	Maximum	Mean		
					Statistic	Std. Error	Std. deviation
Preterm < 37 weeks	Day 5	30	24	208	90.17	8.697	47.63
	Day 15	30	25	146	75.97	5.368	29.40
Full-term 40±2 weeks	Day 5	30	7	182	74.33	6.665	36.5
	Day 15	30	11	119	76	4.565	25

The breast milk CFE in the 25 to 35-year-old mothers significantly elevated 15 days after childbirth in comparison to that in the 35 to 45-year-old mothers (P=0.01). Based on the obtained data, on day 5, the mean breast milk CFEs in mothers with preterm and full-term delivery were 123.33±60.55 and 105.33±51.7, respectively. These values were 95.85±32.47 and 88.27±31.1 in the mentioned groups on day 15, respectively.

Although the comparison of CFE in the breast milk samples obtained on days 5 and 15 showed no statistically significant difference, our investigations revealed a significant correlation between CFE on days 5 and 15 (Table 2). Moreover, our findings demonstrated that the CFE of breast milk stem/progenitor cells of mothers with preterm delivery were higher than that in the mothers with full-term delivery (Table 3).

Discussion

Not all aspects of human breast milk are understood, the nutritional and cellular contents of this substance are under investigation. The anti-infective characteristics, along with the optimal balance of proteins and immunoglobulins, essential polyunsaturated fatty acids, vitamins, and carbohydrates, have been recommended for mature (term) and premature (pre-term) infants (19, 20). Even though the nutritional composition of human breast milk is well examined, our knowledge of breast milk stem/progenitor cells needs more investigation. Recent observations uncovered that human breast milk has the heterogeneous composition of neuroepithelial and myoepithelial lineage, immune cells, mesenchymal stem cells, and hematopoietic stem/progenitor cells (21, 22).

In the present study, we evaluated the CFE of breast milk stem/progenitor cells of mothers with full-term and preterm delivery. The development of infant's organs, such as gastrointestinal and immune systems, initiates in the uterus, while the postnatal development continues by breastfeeding (23). The organ development in preterm infants had a higher reliance on breastfeeding than that in term infants because they are born prematurely. We evaluated the CFE of mother's breast milk stem/progenitor cells on days 5 and 15 after delivery. Totally, our results revealed that CFE on day 5 was higher than that on day 15. In addition, the CFE of mother's breast milk stem/progenitor cells with preterm infants was higher than that in full-term infants.

The solid evidence illustrates that the colostrum (also known as beestings or first milk) has a high concentration of supporting factors, including immunoglobulins, cellular stimulatory factors, proteins, and oligosaccharides, which are necessary for preterm and term infants (24, 25). Colostrum is commonly produced on the first 3-5 days after childbirth (26). Based on our obtained data, it can be stated that the elevated CFE on day 5 in the breast milk of mother with both full-term and preterm infants is due to the presence of colostrum stimulatory factors. On the other hand, the proliferation-inducing factors emerged in the colostrum are thought to be promoting the expansion of breast milk stem/progenitor cells, which are basically required for newborn's organ development (25, 27).

Our results showed that the CFE was higher in mother's breast milk stem/progenitor cells with preterm delivery than in those with full-term delivery. The accumulated evidence illustrates that the breast milk of mothers with preterm infants had a higher amount of cholesterol, phospholipids, easily absorbed medium-chain with 14-18 carbons, and polyunsaturated free

fatty acids with 20-22 carbons chain than that of mothers with full-term delivery (28).

The fatty acids are involved in the cell membrane and may also serve as the source of expansion and myelinization of the brain cells and nervous system in the newborns. These specific products reflect the unique biochemical and physiological needs of preterm infants. Accordingly, it can be inferred that the increased CFE, along with the different types of fat, are the homeostatic strategy to compensate the organ's immaturity in preterm infants.

Crago et al. revealed that 40-60% of the uncultured fresh human breast milk cells contained leukocytes (29). The leukocytes in human breast milk are transported from neonate's intestine to the blood circulation system and integrate into the organs (17, 23). It has been suggested that the breast milk stem/progenitor cells follow a similar mechanism. They differentiate into required cell types and integrate into various tissues. These stem/progenitor cells are potentially releasing essential factors and contributing to the development, regeneration, and homeostasis of preterm infant's tissues. Moreover, these stem/progenitor cells can potentially be responsible for the conservative effects of mother's breast milk against infant's diseases (16).

Further investigations revealed a correlation between prenatal mortality, intrauterine death of fetus and premature delivery in childbearing females and gestational age in mothers aged > 40 years (30). (In line with our results, the prevalence of preterm birth elevated with advancing maternal age. The level of prenatal death and premature delivery underwent a rise with increasing the age and delaying the pregnancy complications after the age of 35 years (31). The 35 to 45-year-old mothers are faced with various risk factors, including hypertension, antepartum hemorrhage, placenta praevia, multiple gestations, prelabor rupture of the membranes, and preterm labor (30, 32).

In the anatomical prospect, an increase in breast size, commonly due to the expansion of the adipose tissue, are clearly fueled by ovarian hormones, including estrogen and progesterone (33). Indeed, the growth hormones, along with ovarian hormones and prolactin, play a notable role in the expansion of breast milk stem cell population not only in adolescence but also during fetal life (34, 35).

Generally, the mammary ducts and/or lobules are developed at the end of each menstrual cycle; therefore, this phenomenon gradually continues during adult life until approximately 35 years of age. However, although accumulated evidence shows that progression in maternal age increases the adverse pregnancy outcomes, from the perspective of clinicians, there is no approved evidence indicating that mothers aged ≥ 35 years have an increased risk of delivering neonates with disorders.

To the best of our knowledge, we believe that the elevated level of CFE in the older mothers may be associated with the reduction of risk factors threating infant's health, which neutralizes the negative point of pregnancy in older mothers. In the present study, we evaluated the CFE in the breast milk of mothers with preterm and full-term delivery on days 5 and 15 after childbirth.

The obtained evidence demonstrates that CFE elevated in mothers' breast milk with preterm delivery in comparison to that in mothers with full-term delivery. The CFE was higher on day 5 after delivery than on day 15 which may be due to the colostrum stimulatory factors. Based on our investigations, CFE of their mothers' breast milk stem/progenitor cells showed no significant correlation with infant's gender, height, and head circumference. There was also a significant relationship between the infants' weight (3,000-4,000 g) and CFE of their mothers' breast milk.

Conclusion

Based on the evaluated results, CFE increased in the breast milk of mothers aged ≤ 35 years in comparison to that in the younger mothers. The CFE was higher in mothers' breast milk with preterm delivery than in mothers' breast milk with full-term delivery. These observations may uncover the compensatory mechanisms illustrated in the mothers' breast milk to improve the preterm infants' tissue development. The pilot nature of our study and reduced parental participation may consider our limitations. However, more participants and variables will be needed in future investigations.

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Conflicts of interests

There are no conflicts of interest.

Authors' Contributions

Dr. Ahmad Shah Farhat designed and supervised the experiments, analyzed the data, and edited the paper. Dr. Daryoush Hamidi Alamdari designed the experiments. Abolfazl Nosrati Tirkani designed and performed the experiments, analyzed the data, and wrote and edited the paper. Mohammad Hasan Arjmand designed the experiments. Mohammad Jalili-Nik analyzed data and wrote the paper.

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