

ORIGINAL ARTICLE

Immune-related miRNAs (181a, 146, 150) Expression in Colostrum and Mature Maternal Milk in normally and Caesarean Delivered Females

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ABSTRACT

Key words:

Colostrum, mature milk, miRNAs, vaginal delivery, cesarean section

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Background: Breast milk is rich in microRNAs that is important for human growth, development, and immune-mediated protection. The method of delivery could alter the wide range of compounds and bioactive molecules present in the human breast milk. **Objectives:** in this work we studied the expression of the immune-related miRNAs (miR-181a, miR-150, and miR-146) in colostrum and mature human milk in normally delivered and cesarean section delivered females to assess the delivery method impact on their expression levels. **Methodology:** a cross-section study included 32 females; 16 delivered by normal vaginal delivery and 16 by cesarean section. Colostrum and mature milk were obtained from each female and the expression level of miR-181a, miR-150, and miR-146 were assessed. **Results:** The expression of miR-181a and miR-146 were significantly higher in colostrum than mature breast milk among all participants (P 0.008, 0.020) and among normal vaginal delivery women (P 0.019, 0.045). While only miR-181a was significantly expressed in colostrum than mature breast milk of cesarean section delivered women (P 0.012). **Conclusion:** The immune-related miRNAs (miR-181a, miR-146 and miR-150) are variably expressed in colostrum and mature milk of mothers gave birth by different methods of delivery. Thus, feeding newborns with all breast milk stages is essential to help their immune system development and maturation and the mode of delivery affects the bioactive molecules profiles in human breast milk.

INTRODUCTION

Natural childbirth –unassisted and assisted– and delivery by cesarean surgery are the available options for delivering a baby. The natural unassisted vaginal delivery is going on without any induction, anesthesia, or episiotomy.¹ While, in caesarean section delivering the baby is done by a surgical intervention. Cesarean section is primarily performed as a live-saving procedure if the mother's or baby's health will be at significant risk during vaginal delivery.²

According to the World Health Organization (WHO), the ideal rate of caesarean delivery is 10 - 15% in order to provide a significant reduction of both maternal and fetal morbidity and mortality.³ On the other hand, cesarean section carries the risk of anesthesia complications, hemorrhage and infections, as a major surgical procedure, in addition to the risk of

neonatal respiratory distress compared to natural childbirth.¹

The process of feeding the baby from delivery until the age of two years directly from a woman's breast is considered breastfeeding. The World Health Organization (WHO) reported that feeding a newborn during the first six months with breast milk alone without any additional external supplements –absolute breastfeeding– provides the infant with optimal nutrition with the beneficial outcomes on health and immunity.⁴ During lactation, human breast milk passes through three stages; starting with colostrum that is the milk expressed within the first 7 days after delivery, transitional milk is the milk expressed 7–14 days after birth, and mature milk that is lactated from 2 weeks after birth onwards. It has been proved that the nutritional components of colostrum and mature milk are evidently different.⁵

Human breast milk is a complex liquid containing all bioactive molecules sufficient for the infants' nutrition and development of immune system.⁶ It is also rich in microRNAs –a small non-coding RNA molecules– which are known to be important for human development and protection.⁷ Although the exact role of miRNAs in human milk is still unclear, it is thought that they are important for growth, development, and immune-mediated protection for the newly born baby.⁸ Studies revealed that miRNAs are significantly involved in immune regulation functions including immune cells development, maturation and differentiation as well as the associated immune response.^{9,10}

More than 1400 miRNAs had been identified in human milk including miRNAs that known to have some immunological roles such as miR-17, miR-92a, miR-125b, miR-146, miR-150, miR-155, miR-181a and miR-223.¹¹⁻¹³ For instance, miR-181a acts as an intrinsic modulator of T-cell selection and sensitivity to specific antigens.¹⁴ It has been shown that miR-181a expression is changing during different T cell development stages in thymus and T cell differentiation in lymph nodes. It shows higher expression in thymus, to help thymic development of conventional T, regulatory T, iNKT (Invariant natural killer T) and MAIT (mucosal associated invariant T) cells by decreasing TCR (T-cell receptor) sensitivity and activation thresholds via suppressing some phosphatases. While its lower expression in periphery enhances TCR sensitivity to antigens and stimulates peripheral T cell activation.¹⁵

Studies showed that miR-146a is linked to the regulation pathways of both adaptive and innate immune responses.¹⁶ by promoting cell differentiation and regulating toll-like receptors recognizing PAMPs and cytokine signaling, respectively. It has been also shown that miRNA-146a indirectly reduces the expression of NF- κ B leading to and participating in inflammatory reactions through the body.¹⁷ Regarding miR-150, it has been found to control B cell differentiation and is crucial for pre- and pro-B cell differentiation and effector function.¹⁸ It is known to have a suppressor effect on B cells when expressed prematurely in human breast milk through targeting the transcription factor c-Myb and blocking the early B-cell development.¹⁹ miR-150 has also an anti-inflammatory effect through the down regulation of STAT1 in macrophages.²⁰

Thus, we studied the miRNAs miR-181a, miR-150, and miR-146 expression in colostrum and mature human milk in normally delivered and cesarean section delivered females to assess the delivery method impact on the expression levels of the studied miRNAs.

METHODOLOGY

Study design:

The current cross-section was conducted on a total of 32 females attended the Obstetrics and Gynecology Department at Benha University Hospitals for delivery between February and May 2023. Sixteen females gave birth through normal vaginal delivery and 16 females delivered by cesarean section. All included participants were apparently healthy, aged between 20 and 40 years, had a full-term pregnancy (≥ 38 weeks of gestation) and delivered a single live-birth baby. None of them were smokers or alcoholic, on antioxidant vitamins or elemental supplements or suffering from any acute or chronic health conditions.

Laboratory work was performed in Medical Microbiology and Immunology Department, Faculty of Medicine, Benha University. The ethical approval of the study protocol was obtained from the Ethical Scientific Committee, Benha Faculty of Medicine (RC.26-1-2023). All participants signed an informed consent before enrollment in the study.

Samples & samples preparation:

Each participant gave 2.0 ml colostrum between 1–7 days after delivery and 2.0 ml mature milk any time after 14 days of delivery. All samples were taken by manual expression for a single breast in a sterile 5-ml Falcon polypropylene tubes. Samples were stored immediately at -80°C . Just before testing, samples were thawed on ice, centrifuged two times ($1200\times g$ for 10 min at 4°C) to remove fat, cells and large debris. The obtained supernatants were centrifuged at maximum speed (for 30 min at 4°C) to remove any residual fat or protein and the clear supernatant was taken.

Methods:

Total RNA including miRNA was extracted from the clear supernatant of prepared milk samples using EasyPure[®] miRNA Kit (Transgen Biotech, China) according to manufacturer's instructions. Both quantity and purity of the extracted RNA were assessed spectrophotometrically by NanoDrop 2000 (Thermo Fisher Scientific, USA). The cDNA was produced by reverse transcription (RT) using the miRCURY LNA RT Kit (cat. no. 339340) (QIAGEN, Germany). For each 20 μ l RT reaction, 4 μ l miRCURY SYBR[®] Green RT Reaction Buffer, 10 μ l RNase-free water, 2 μ l miRCURY RT Enzyme Mix and 4 μ l template RNA (5 ng/ μ l) were added. The RT reaction was done by incubation for 60 min at 42°C followed by 5 min at 95°C then a final hold at 4°C . For accurate and reproducible miRNA quantification, an equal amount of *Caenorhabditis elegans* miRNA (cel-miR-39) was spiked in each sample as internal reference for normalization of the target miRNAs.

Quantitative real time – polymerase chain reaction (qRT-PCR) was performed for the studied miRNAs (miR-181a, miR-150, and miR-146) and (cel-miR-39) using the miRCURY LNA miRNA SYBR® Green PCR Kit (cat. no. 339345) and miRCURY LNA miRNA PCR Assays (cat. no. 339306) (QIAGEN, Germany) according to manufacturer's protocol. For each 10µl PCR reaction mix, 5µl miRCURY SYBR® Green Master Mix, 1µl RNase-free water, 0.4 µM miRNA specific forward primer (Table 1), 0.4 µM universal reverse primer (D352) and 3µl cDNA template were added. PCR was run on Roter-Gene Q (QIAGEN, Germany) under the following conditions: 95°C for 2 min, followed by 45 cycles of 95°C for 10 sec and 56°C for 1 min with final hold at 4°C. Quantification of the targeted miRNAs were expressed as relative units (RU) after being normalized to cel-miR-39 according to the $2^{-\Delta\Delta ct}$ method.

Table 1: miRNAs primers' sequences

miRNA	Forward primer
miR-181a	5'-GCGGTAACATTCAACGCTGTGCG-3'
miR-150	5'-TCTCCCAACCCCTTGTACCAGTG-3'
miR-146	5'-AACATTCAACGCTGTGCGGTGA-3'
cel-miR-39	5'-GGTCCGTGTAAATCAGCTT-3'

Statistical analysis:

IBM® SPSS® vs. 23 (NY, USA) was used for data administration and statistical analysis. Quantitative data were represented as mean and standard deviation (SD) while qualitative data as number and frequency. Means of miRNAs expression levels were compared using student t-test. The accepted level of significance in this work was 0.05.

RESULTS

Participant characteristics:

This study included 32 females with mean age 30.25±3.56 years. They were apparently healthy and delivered full-term, single healthy newborns, either

through normal vaginal delivery (16 females) or by uncomplicated Cesarean section (16 females). The demographic and clinical characteristics of study participants are summarized in Table 2.

Table 2: Demographic and clinical characteristics of studied participants

Characteristic		Value
Age (years)		30.25 ± 3.56
Delivery Method	Cesarean section	16 (50%)
	Normal Vaginal	16 (50%)
Gestational age at birth (weeks)	38	4 (12.5%)
	39	6 (18.75%)
	40	14 (43.75%)
	41	6 (18.75%)
	42	2 (6.25%)
Mother BMI (kg / m ²)		22 ± 2.1
Mother height (cm)		165.44 ± 5.69
Mother weight (kg)		62.4 ± 7.5
Parity	Primiparous	22 (68.75%)
	Multiparous	10 (31.25%)
Education	Lower	18 (56.25%)
	Higher	14 (43.75%)
Occupation	Housewife	12 (37.5%)
	Working	20 (62.5%)

Data represented either mean ± SD or number (frequency).

miRNAs expression in colostrum and mature breast milk:

The expression levels of the studied miRNAs in colostrum and mature human milk obtained from all participants showed significant higher expression of miR-181a and miR-146 in colostrum compared to mature breast milk (P 0.008 and 0.020, respectively) while miR-150 expression in colostrum is higher than in mature breast milk without reaching significance. (Table 3)

Table 3: Comparison of miRNAs expression between colostrum and mature milk in all studied participants

miRNAs	Colostrum	Mature milk	<i>t</i>	P-value
miR-181a	1.71±0.225	1.49±0.225	2.832	0.008
miR-146	1.53±0.178	1.38±0.168	2.451	0.020
miR-150	1.33±0.249	1.21±0.292	1.237	0.226

miRNAs expression in colostrum and mature breast milk in different delivery methods:

Comparing the studied miRNAs expression among women delivered their babies via normal vaginal delivery, revealed that miR-181a and miR-146 were

significantly highly expressed in colostrum than mature breast milk (P 0.019 and 0.045, respectively). While, among women delivered by cesarean section, only miR-181a was significantly highly expressed in colostrum than mature breast milk (P 0.012). (Table 4, Fig. 1 & 2)

Table 4: Comparison of miRNAs expression between colostrum and mature breast milk in different delivery methods

Delivery Method	miRNAs	Colostrum	Mature Milk	<i>t</i>	P-value
Normal Vaginal	miR-181a	1.55±0.141	1.34±0.177	2.655	0.019
	miR-146	1.45±0.141	1.3±0.131	2.201	0.045
	miR-150	1.2±0.185	1.16±0.338	0.275	0.787
Cesarean Section	miR-181a	1.88±0.167	1.64±0.159	2.907	0.012
	miR-146	1.61±0.181	1.46±0.169	1.717	0.108
	miR-150	1.46±0.245	1.26±0.25	1.616	0.128

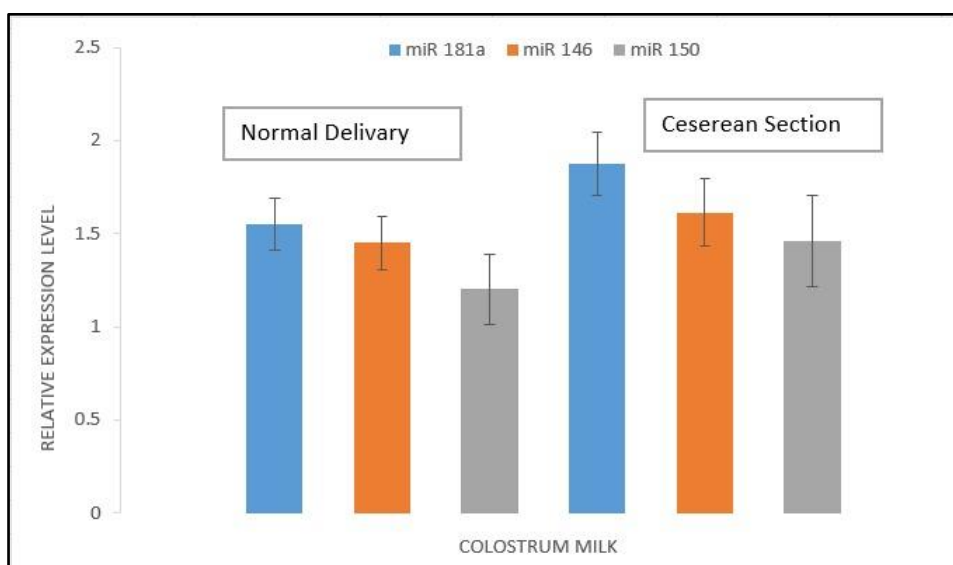


Fig. 1: Differential expression of studied miRNAs in colostrum milk according to delivery method

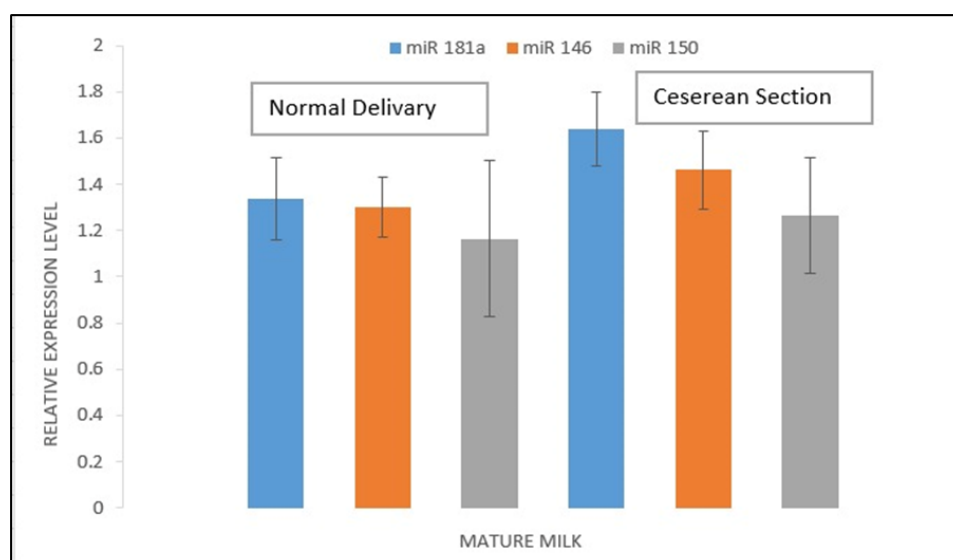


Fig. 2: Differential expression of studied miRNAs in mature milk according to delivery method

DISCUSSION

Breast milk is the essential nutritional source for the infants. However, its main function early after birth is to help the immune system development, maturation and differentiation as well as supporting the growth, and development of the newborn. Thus, the composition of the early produced colostrum differs from transitional and mature milk, as it contains vast amount of physiological, immunological and immunomodulatory molecules.²¹ Among those factors, miRNAs that have been isolated from human breast milk.²²

Several studies had investigated the role and effect of milk expressed miRNAs on immune system and immune-related conditions. They showed the involvement of several miRNAs expressed in human milk in immune modulatory pathways²³, such as the role of miR-155 in the development and maturation of regulatory T cells (Treg)²⁴ and the effect of miR-375-3p consumption in atopy risk reduction²⁵. While miR-148a-3p and miR-155-5p/miR-29b-5p in human breast milk were found to support B-cell proliferation in infants during breastfeeding through the upregulation of BCL6.²⁶

Studies revealed that the composition of human milk is affected by multiple factors in both mother and baby such as maternal age, diet, health conditions, newborn sex, gestational stage (preterm or term) and lactation stage.²⁷ Some of these factors might affect the microRNA profile of different stages of human breast milk.²⁸ A number of certain miRNAs have been proved to control different aspects of immune system and immune responses²⁹ however their exact roles in breast milk still not fully elucidated.

Here we studied the expression of miR-181a, miR-146 and miR-150 in colostrum and mature breast milk in women gave birth by either normal vaginal delivery or cesarean section.

We found that the expression levels of miR-181a and miR-146 were significantly higher in colostrum compared to mature breast milk (P 0.008 and 0.020, respectively).

This has been proved previously by Kosaka and his collages¹¹. While another study has reported the presence of miR-181a along with miR-223 in colostrum and human milk suggesting their role in regulating T cells and granulocytes responses towards selected targets.³⁰ Also, it was shown that the presence of miR-146 in colostrum plays a significant role in modulating TLRs signaling thus protect the baby from a variety of diseases, including cancer.³¹

We also found that colostrum is not rich in miR-150 which suppresses B-cell differentiation. This finding aligns with previously published report.¹¹

We investigated the effect of delivery method (normal vaginal or cesarean section) on the studied

miRNAs in colostrum and mature breast milk in order to test the hypothesis that the delivery method may be related to altered expression of immune related microRNAs in breast milk.⁸

We found that miR-181a and miR-146 showed significant higher expression in colostrum than mature breast milk (P 0.019 and 0.045, respectively) among women delivered by normal vaginal delivery and only miR-181a showed significant higher expression in colostrum than mature breast milk (P 0.012) among women delivered by cesarean section.

To the best of our knowledge, this is the first study that investigated those miRNAs expression in different breast milk stages regarding the method of delivery. However, the expression of other miRNAs such as miR-148a and miR-125b in different human breast milk stages in relation to the delivery method have been published.³² As they found those miRNAs were significantly reduced in mature breast milk in mothers delivered by caesarean section compared to exosome microRNA levels observed in normal vaginal delivery.³² Also, Słyk-Gulewska et al. reported an altered expression of microRNA molecules in milk of mothers gave birth by caesarean section.²⁶

Moreover, the increased levels of oxytocin during vaginal delivery was shown to be responsible for the increased levels of certain microRNAs in human colostrum rather than other breast milk stages.³³

Furthermore, some inflammatory conditions reported in newborns, infants, children and even adults have been linked to the cesarean section that is considered to be one of the environmental risk factors for chronic immune and inflammatory diseases. Therefore, various immune related miRNAs are supposed to be upregulated in the colostrum of mothers who gave birth by cesarean section to regulate the inflammatory responses and to boost the immune defense mechanisms of the newborn.^{11,34,35}

The present study was a small-scale investigation. Larger studies need to be conducted to investigate the expression level of the immune-related miRNAs associated with inflammatory response and autoimmune diseases in human breast milk for further clarification of the effect of delivery method on the pathogenesis of immune-related diseases.

CONCLUSION

The immune-related miRNAs (miR-181a, miR-146 and miR-150) that have a significant role in innate and adaptive immune response development, maturation and function are differentially expressed in colostrum and mature breast milk of mothers gave birth by different methods of delivery. Thus, we could conclude that feeding newborns with all breast milk stages is essential to help their immune system development and

maturation and the mode of delivery affects –a long with other factors– the bioactive molecules profiles in human breast milk.

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article. This manuscript has not been previously published and is not under consideration in another journal.

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