Title page

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Title: Highest concentration of breast milk-derived exosomes in colostrum

Running title: Breast milk-derived exosome in colostrum

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Disclosure

Masaki Terahara and Yoshitaka Nakamura are employed by Meiji Co., Ltd. Japan. The other authors declare no conflict of interest.

Abstract

Background: Exosomes are nanosized extracellular vesicles, that play important roles in intercellular immune regulation. They have potential therapeutic utility for neonatal diseases including necrotizing enterocolitis. Breast milk-derived exosomes have recently shown beneficial effects on intestinal damage *in vitro* and *in vivo*. However, the chronological change in breast milk-derived exosome concentrations after delivery are unclear.

Methods: In this prospective study, we enrolled 17 mothers who delivered premature infants admitted to a neonatal intensive care unit in Japan. We measured the consecutive concentrations of breast milk-derived exosomes in the mothers for 48 weeks after delivery.

Results: The median concentration of breast milk-derived exosomes was 1.62×10^{8} particles/ml in colostrum, showing a significant decrease after 2 weeks ($p < 0.01$). There was no association between the exosome concentration in colostrum and maternal perinatal factors including parity, mode of delivery, maternal age, and gestational age at delivery.

Conclusions: We concluded that breast milk-derived exosomes were the richest in colostrum. Our basic data of breast milk-derived exosomes are expected to aid in the clinical application of exosomes for treating neonatal diseases.

Key words: breast milk, colostrum, exosome, neonate, preterm

1. Introduction

Exosomes are the smallest vesicles in the extracellular vesicle family,[1] exerting crucial physiological effects on intercellular immune regulation, organ development, and reproductive performances.[2,3] Extracellular vesicles-related therapeutics have been developed and applied for the treatment of inflammatory diseases.[3] It has also been discussed that extracellular vesicles have potential therapeutic utility for neonatal diseases such as hypoxic ischemic encephalopathy, retinopathy of prematurity, spina bifida, and necrotizing enterocolitis (NEC).[4] Exosomes are observed in all human body fluids, including saliva, plasma, urine, and breast milk, the last of which is known to contain high concentrations of exosomes.[1,5,6]

Breast milk is the most beneficial food for infants.[7] Recent evidence has shown that an exclusive diet of human milk decreases the incidence and severity of NEC, a devastating inflammatory bowel disease of premature infants.[7-9] Several basic studies have demonstrated the beneficial effects of breast milk-derived exosomes against NEC *in vitro* and *in vivo*.[10-12] However, while breast milk is rich in exosomes with early milk containing a higher concentration than mature milk,[13] studies on the chronological change in breast milk-derived exosome concentrations after delivery are limited.

Therefore, we prospectively evaluate the change in the concentration of breast

milk-derived exosomes obtained from mothers who delivered preterm infants for 12 months after delivery. In addition, we also investigated the association between perinatal factors and the concentration of breast milk-derived exosomes in colostrum.

2. Methods

2.1 Ethical Statement

This prospective observational study protocol was approved by the Institutional Review Board of Shiga University of Medical Science (approval number 27-134). We used breast milk samples after obtaining written informed consent from mothers whose infants were admitted to our hospital.

2.2 Subjects

The subjects of the study were selected from mothers to infants with a birth weight over 1500 g admitted in the neonatal intensive-care unit (NICU) of Shiga University of Medical Science Hospital between January 2016 and July 2016. We obtained written informed consent for their participation in the study within 24 h after delivery. We excluded subjects who refused to sign the informed consent form. We did not enroll

mothers whose infant's length of hospital stay was expected to be less than four weeks after their birth or whose infants had congenital malformations.

2.3 Sample Collection

The breast milk was collected at birth and 2, 4, 8, 24, and 48 weeks after delivery. The collected breast milk samples were immediately frozen and stored at −60 ℃. We defined breast milk first obtained within 72 h after delivery as the colostrum.

2.4 Extraction of Exosomes

Each breast milk sample was centrifuged at 2,000 × g for 10 minutes at room temperature to remove cells and debris. The supernatant was transferred to a new tube, and then centrifuged at 10,000 × g for 30 minutes at room temperature to remove micro debris. The supernatant containing the clarified whey was then transferred to a new tube, and 200 µl of clarified whey was re-suspended in 200 µl of phosphate-buffered saline. The exosomes were isolated using 100 µl of Total Exosome Isolation Reagent (Thermo Fisher, USA) according to the manufacturer’s instruction.

2.5 Quantification of Exosomes

Taking 20 µl of the exosomes fraction and 80 µl of lysis buffer of EXOCET (SBI, USA), the total sample was incubated for 5 minutes at 37 ℃ and then centrifuged at 1,500 × g for 5 minutes at room temperature. The supernatant was transferred to a new tube, and 50 µl of reaction buffer from the same kit was added, followed by incubation for 20 minutes at room temperature. A standard curve was generated according to the description of the kit. The absorbances were measured using iMARK micro plate reader (Bio Rad, USA) immediately at 405 nm, according to the manufacturer's instructions.

2.6 Statistical Analyses

Differences in exosome concentrations between at delivery and at each measurement time point after delivery were tested by a repeated-measures one-way analysis of variance followed by Dunnett's multiple comparison test. The impact of perinatal factors on the concentration of breast milk-derived exosomes in colostrum was estimated using Mann–Whitney *U*- test.

All statistical analyses were performed by using the Bell Curve for Excel software program (Social Survey Research Information Co., Ltd., Tokyo, Japan). A *P* value of <0.05 indicated a significant difference.

3. Results

3.1 Background Characteristics

As shown in Fig. 1, 89 infants with a birth weight of more than 1500 g were admitted to the Shiga University of Medical Science Hospital's NICU. Sixty-seven were not enrolled in the present study because of short-term hospital stay, refusal of participation, or congenital malformation syndrome. A total of 22 infants were included in the study, and the number of mothers was 18 because of 4 pairs of twins. We analyzed 17 mothers` breast milk, excluding one mother with a twin birth because of failure to collect clarified whey of colostrum.

Table 1 shows the background characteristics. The median maternal age and gestational age at birth were 33 years old and 33.8 weeks, respectively. All of the deliveries were preterm (< 37 weeks). Eight cases (47%) were primipara, 12 (71%) were Caesarean section, and 3 (18%) were twins.

3.2 Chronological Changes in Breast Milk-derived Exosome Concentrations

The median concentrations of breast milk-derived exosomes were 1.62, 0.45, 0.38, 0.29, 0.26, and 0.64×10^{8} particles/ml at 0, 2, 4, 8, 24, and 48 weeks after delivery, respectively (Fig.2). There were significant decreases in the exosome concentrations at 2, 4, 8, 24, and

48weeks after delivery compared to colostrum (* $p<0.01$). There was no significant difference in the exosome concentrations in breast milk obtained 2 to 48 weeks after delivery.

3.3 Perinatal Factors Influencing the Concentration of Breast Milk-derived Exosomes in Colostrum

We evaluated the effect of maternal perinatal factors including parity, mode of delivery, maternal age, gestational age at delivery, hypertensive disorders of pregnancy, chorioamnionitis, and gestational diabetes mellitus on the concentration of exosomes in colostrum. There were no significant differences in breast milk-derived exosome concentrations at birth based on those perinatal factors (Table 2)

4. Discussion

In the current study, we found that the concentration of breast milk-derived exosomes was the highest at delivery with a rapid decrease after delivery. In addition, the concentration of breast milk-derived exosomes at delivery was not associated with maternal perinatal factors at delivery.

The concentration of breast milk-derived exosomes was the highest at delivery,

showing a rapid decrease after delivery. Our result is consistent with the findings of previous studies showing that the exosomes derived from colostrum are richer than those from transitional and mature milk.[12,13] However, there are several differences between the previous study and ours with regard to comparing the concentrations of colostrum and mature milk. Torregrosa et al. collected mature milk at 2 months after delivery and compared it with colostrum.[13] It is difficult to show when the concentration of exosomes in colostrum decreases after delivery. As our mature milk samples were collected in the same periods after birth, we were able to demonstrate in detail the time when a rapid decrease in the concentration of breast milk-derived exosomes occurred and also demonstrate the richest exosomes in the colostrum. Gao et al. compared unpaired samples obtained from different mothers in each lactation period, whereas we compared samples from the same mothers in each period.[12] Compared to previous studies, our data are considered much more accurate for the assessment of exosome concentrations in various lactation periods. In addition, the concentration of exosomes in colostrum in our study was approximately three times higher than that in mature milk, while it was approximately 40 % higher in Gao's study than in mature milk.[12] The differences in exosome concentrations in colostrum compared to mature milk among these two studies may be due to differences in the sample collection methods.

Next, the concentration of breast milk-derived exosomes at delivery was not associated with maternal perinatal factors. To our knowledge, this is the first time this finding has been reported. Recent studies have demonstrated the ability of human breast milk-derived exosomes to protect against intestinal damage.[11,12,14] Our finding may support the ability of colostrum, which is rich in exosomes, to reduce intestinal damage more effectively than mature milk. One possible explanation for the differences in exosome concentrations between colostrum and mature milk is due to the changes in cell populations and/or the cellular phenotype of breast milk.[13,15] Our previous study demonstrated that the earlier the gestational age of infants at birth, the lower the concentration of serum-derived exosomes.[16] If preterm infants can consume exosomes through maternal breast milk, it is reasonable that colostrum would be more beneficial to immature infants in terms of protecting against intestinal damage than in mature infants. Further studies regarding breast milk-derived exosomes are needed.

Several limitations associated with the present study warrant mention. First, we did not demonstrate the presence of exosomes by their positive marker in our samples due to the limited amount of breast milk available. Several studies have confirmed the presence of exosomes by Western blotting for the positive marker CD63.[11,17] Although we used the data without confirming this presence, we were still able to evaluate the time

course of the exosome concentration in breast milk. Second, we were unable to measure the exosome concentration multiple times due to the limited sample volume. Our data might therefore have been less accurate than with more than a single measurement. Third, we analyzed the data with small number of samples. The relationship between breast milk-derived exosome concentrations and maternal perinatal factors may not be fully evaluated. Additionally, further case series and studies at less than 35 weeks are warranted.

5. Conclusions

We concluded that the concentration of breast milk-derived exosomes was the highest in colostrum, showing a rapid decrease after 2 weeks from delivery. Our basic data on breast milk-derived exosomes are expected to prove useful for the clinical practice of treating neonatal diseases.

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Disclosure

Masaki Terahara and Yoshitaka Nakamura are employed by Meiji Co., Ltd. Japan. The other authors declare no conflict of interest.

Author contribution

M.O., S.K., R.I., T.T., M.T. and Y.N. designed the study; M.O., S.K., I.J. and M.O. collected data; M.O., S.K., R.I., T.T., M.T. and Y.N. analyzed data; M.O. and S.K. wrote the manuscript; R.I., T.T., M.T., Y.N. and Y.M. revised the article. All authors read and approved the final manuscript.

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Figure legend

Figure 1. Flow chart of this study

Figure 2. Chronological change in the concentration of breast milk-derived exosomes (n=17). Horizontal lines represent the median values. Compared to the colostrum, significant differences were noted at 2, 4, 8, 24 and 48 weeks after delivery (* $p<0.05$).

Table 1. Characteristics

Maternal information	**n=17**
Maternal age at birth (years)	33 (29.0-35.0)
Primipara	8 (47)
Gestational age at birth (weeks)	33.8 (33.1-35.1)
Mode of delivery; Caesarian section	12 (71)
Multiple birth; twin	3 (18)

Data are presented as the median (interquartile range) or n (%)

Table 2. Impact of perinatal factors on exosome concentrations in colostrum

Perinatal factor	**Concentration ($\times 10^8$particle/ml)**	**Difference †**
Parity		N.S.
Primi (n=8)	1.39 (1.17-1.91)	
Multi (n=9)	1.64 (0.94-2.69)	
Mode of delivery		N.S.
Vaginal (n=5)	1.04 (0.94-1.64)	
Caesarian (n=12)	1.67 (1.17-2.70)	
Maternal age (years)		N.S.
<35 (n=10)	1.59 (1.24-2.43)	
≥35 (n=7)	1.04 (0.83-2.24)	
Gestational age (weeks)		N.S.
<35 (n=11)	1.66 (1.09-2.69)	
≥35 (n=6)	1.14 (0.97-1.47)	

HDP		N.S.
+ (n=2)	0.98 (0.85-1.11)	
- (n=15)	1.63 (0.99-2.69)	
CAM		N.S.
+ (n=7)	1.24 (0.94-2.69)	
- (n=10)	1.59 (1.09-1.76)	
GDM		N.S.
+ (n=3)	0.94 (0.56-1.30)	
- (n=14)	1.59 (1.09-2.69)	

median (interquartile range), † Mann-Whitney U test, HDP, hypertensive disorders of pregnancy; CAM, chorioamnionitis; GDM, gestational diabetes mellitus.