HHS Public Access

Author manuscript

J Pediatr Gastroenterol Nutr. Author manuscript; available in PMC 2020 August 01.

Published in final edited form as:

J Pediatr Gastroenterol Nutr. 2019 August ; 69(2): 235–238. doi:10.1097/MPG.0000000000002363.

Storage of Extracellular Vesicles in Human Milk, and MicroRNA Profiles in Human Milk Exosomes and Infant Formulas

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Abstract

The objectives of this study were to lay the methodological groundwork for field studies of microRNA analysis in exosomes from small sample volumes of human milk, and assess exosome and microRNA content in infant formulas. When human milk was stored at 4°C for four weeks, the count of exosome-sized vesicles decreased progressively to 49% ± 13% of that in fresh milk. Exosomes were purified from 1 mL of fresh human milk and their microRNA content was assessed by microRNA-sequencing analysis and compared to that in infant formulas. We identified 221 microRNAs in exosomes from three samples of fresh human milk; 84 microRNAs were present in all three samples. MicroRNAs were not detectable in infant formulas and their exosome-sized vesicles, which appeared to be casein micelles. We conclude that large scale studies of microRNAs in human milk exosomes are feasible, and exosomes and microRNAs are not detectable in formulas.

Keywords

Bovine milk; exosomes; extracellular vesicles; human milk; infant formula; micro RNA; storage stability; casein; fat globules; protocols; stability

Introduction

Exosomes are extracellular vesicles (EVs) with an important function in cell-to-cell communication ([1], Supplemental Table 1, Supplemental Digital Content 1).

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Authors Roles

Amy Leiferman acquired data, made substantial contributions to the interpretation of data, drafted and revised this work, gave final approval of the work to be published and agreed to be accountable for all aspects of the work. Jiang Shu acquired data, made substantial contributions to the interpretation of data, drafted and revised this work, gave final approval of the work to be published and agreed to be accountable for all aspects of the work. Bijaya Upadhyaya acquired data, made substantial contributions to the interpretation of data, drafted and revised this work, gave final approval of the work to be published and agreed to be accountable for all aspects of the work. Juan Cui acquired data, made substantial contributions to the interpretation of data, drafted and revised this work, gave final approval of the work to be published and agreed to be accountable for all aspects of the work. Janos Zempleni conceived the idea for this work, made substantial contributions to the interpretation of data, drafted and revised this work, gave final approval of the work to be published and agreed to be accountable for all aspects of the work.

Conflicts of Interest: None declared.

Communication is achieved through the transfer of exosome cargos from donor cells to recipient cells. MicroRNA cargos are of particular importance because they regulate the expression of more than 60% of human genes [4]. Exosomes and microRNAs may be absorbed from milk [5]. The objectives of this study were to lay the methodological groundwork for studies of microRNA in exosomes from small sample volumes of human milk in large cohorts of women, and assess exosome and microRNA content in infant formulas. This study is significant because it enhances the confidence that future large scale studies of microRNAs in human milk exosomes are feasible and can contribute new information to the Human Milk Composition Database curated by the United States Department of Agriculture [6].

Methods

Participants

Five healthy non-Hispanic women, ages 26-36 years, participated in this study (1 African, 3 Asians, 1 Caucasian). The women gave birth to healthy singletons and had been lactating for 2-10 months when milk was collected. Two women had given birth to one or two babies before, three women were primiparous. This study was approved by the Institutional Review Board at the University of Nebraska-Lincoln (protocol 14302); written informed consent was obtained from participants.

Storage Stability Analysis

Midstream breast milk was collected. Full-fat bovine milk was purchased from a local grocery store; the date of purchase was denoted time zero but does not represent fresh milk. Aliquots were kept under different conditions [storage at 4°C, as well as −80°C without or with preservatives, glycerol (Sigma-Aldrich, St. Louis, MO) or dimethylsulfoxide (DMSO, Sigma-Aldrich)] in 1.5-mL polypropylene microcentrifuge tubes. At timed intervals samples were diluted with water and exosome-sized vesicles were analyzed using a NanoSight NS300 tracking device (Malvern, Westborough, MA) to assess effects of storage on vesicle count and size (syringe speed, 50; camera level, 10-11; detection threshold, 3; five 1-minute videos per sample).

Exosome Isolation and Authentication

Exosomes were isolated from 1 mL human and bovine milk and three brands of milk-based infant formulas (Supplemental Table 2, Supplemental Digital Content 2) as described previously with minor modifications [7, 8]. Samples were centrifuged at 16,000 *g* for 30 minutes at 4°C to remove fat layer, cells and debris. Fat globules and microvesicles were removed by ultracentrifugation at 83,000 *g* for 60 min at 4°C (Fiberlite F37L-8×100 rotor; ThermoFisher Scientific, Waltham, MA). Exosomes were collected by ultracentrifugation at 130,000 *g* for 90 min and re-suspended in 200 μL PBS. Proteins were extracted from exosomes using ice-cold radioimmunoprecipitation assay buffer (Sigma-Aldrich) with protease inhibitor cocktail (Sigma-Aldrich). Antibodies against CD-9 (Biovision, Milpitas, CA), CD63 (Santa Cruz, Dallas, TX), HSP70 (Santa Cruz) and Alix (Santa Cruz) were used as positive controls; anti-histone H3 (Santa Cruz) was used as a negative control [3]. Antibodies against alpha-tubulin (EMD Millipore, Burlington, MA) and integrin-β (Abcam,

Cambridge, MA) were used to assess contamination with microvesicles. We probed bovine and human caseins in EV preparations from human milk and infant formulas with anti-bovine α-casein (Abcam) and anti-human β-casein (Novus Biologicals, Littleton, CO) [2]. Anti-α-lactalbumin (Abcam) and anti-butyrophilin (Origene, Rockville, MD) were used to probe whey proteins and lipid globules, respectively.

MicroRNA Sequencing

We used protocols endorsed by the Extracellular RNA Consortium [9]. RNA was extracted from human milk exosomes (1 mL milk) using the miRNeasy Micro Kit (Qiagen, Germantown, MD). MicroRNAs were sequenced in the DNA Sequencing Core at the University of Nebraska Medical Center (Omaha, NE). RNA integrity was confirmed using a Fragment Analyzer Automated CE System (Advanced Analytical Technologies, Ankeny, IA). Libraries were prepared using the NEXTflex Small RNA-Seq Kit v3 (Bioo Scientific, Austin, TX) and sequenced using Illumina Hiseq 2500 (~6 million reads/sample). Quality control was performed using FastQC [10]. Adaptor sequences and reads containing ambiguous bases or having an average quality score less than 30 were removed using Cutadapt [11]. Reads were aligned against the human microRNA database, miRBase version 22 using miRDeep2 [12, 13]. Data were deposited in the BioProject database (accession number PRJNA477819).

Quantitative Reverse Transcription PCR (RT-qPCR)

RT-qPCR analysis was performed to assess contamination of spin columns with small RNAs and confirm findings from sequencing analysis. RNeasy MinElute spin columns (Qiagen) might be contaminated with small RNAs, thereby causing artifacts in microRNA analysis [14]. RNeasy MinElute columns were treated with sodium hypochlorite to remove possible contamination [14]. We dissolved a non-natural microRNA (miSpike; IDT DNA, Coralville, IA; 5’-rCrUrCrArGrGrArUrGrGrCrGrGrArGrCrGrGrUrCrU-3’) in 200 μL RNase-free, molecular biology grade water and extracted miSpike using MinElute columns. Eluted miSpike (100 ng) was reverse transcribed using the miScript II RT Kit (Qiagen). In addition, miR-30d-5p, miR-125a-5p and miR-423-5p were analyzed in hypochlorite-treated and untreated columns. RT-qPCR was performed using miScript SYBR Green (Qiagen), the universal reverse primer in the miScript II RT Kit and sequence-specific forward primers (Supplemental Table 3, Supplemental Digital Content 3). Hypochlorite-treated columns were used when confirming sequencing data by RT-qPCR. Melting curve analyses suggested formation of a single product (not shown). Ct values greater than 29 were considered not detectable [15].

Statistical Analyses

Stability data were analyzed using repeated measures ANOVA. Dunnett’s post hoc test was used to compare data from all storage conditions to that of fresh human milk. Vesicle counts in timed samples of stored bovine milk were analyzed by one-way ANOVA and Tukey’s post hoc test for multiple comparisons. R version 3.5.0 (R Foundation for Statistical Computing) was used for analyses. Data are expressed as means ± SD or means ± SEM. Differences were considered significant if $P < 0.05$.

Results

Storage Stability of Exosome-sized Vesicles

Storage caused a loss of exosome-sized vesicles in human milk. When human milk was stored at 4°C, the vesicle count decreased progressively to 99% ± 17% (overnight), 84% ± 27% (2 weeks), and 49% ± 13% (4 weeks, $P = 0.03$) of that in fresh milk (Figure 1A). The losses of exosome-sized vesicles at 4 weeks were not statistically significant in frozen samples or in the presence of preservatives: 71% ± 45%, 59% ± 29% and 78% ± 35% of exosome-sized vesicles were recovered if human milk was stored at −80°C, −80°C with DMSO and −80°C with glycerol, respectively. We conducted a power calculation ($\alpha = 0.05$, $\beta = 0.8$) using the mean ± SD of exosome-sized vesicles in fresh milk and the null hypothesis that storage does not affect the count of exosome-sized vesicles in human milk. One hundred forty-eight samples are needed to detect a 25% loss of vesicles. The moderate increases in vesicle size during storage were not statistically significant (Figure 1B, C). Storage did not affect the count and size of exosome-sized vesicles in bovine milk (Supplemental Figure 1; Supplemental Digital Content 4).

Exosome Authentication

Vesicles purified from human milk are exosomes, whereas those from formulas are mostly casein micelles. Human milk contained $2.18 \pm 1.53 \times 10^{11}$ vesicles per mL with mean and modal sizes of 149.7 ± 20.7 nm and 126.7 ± 6.7 nm, respectively (n = 3). Formula 1 contained $2.72 \pm 0.13 \times 10^{10}$ vesicles per mL with mean and modal sizes of 140.8 ± 4.5 nm and 128.6 ± 9.6 nm, respectively (n = 3). Formula 2 contained $1.22 \pm 0.08 \times 10^{11}$ vesicles per mL with mean and modal sizes of 199.8 ± 4.5 nm and 170.6 ± 22.6 nm, respectively (n =3). Formula 3 contained $2.14 \pm 0.22 \times 10^{10}$ vesicles per mL with mean and modal sizes of 142.7 ± 7.2 nm and 118.6 ± 6.0 nm, respectively (n = 3). Exosomes purified from human milk tested positive for CD9, CD63, and Alix, and negative for histone H3 and alpha-tubulin (Supplemental Figure 2A, B; Supplemental Digital Content 5). There was little evidence of contamination with EVs larger than expected for exosomes (Supplemental Figure 2C). The vesicles purified from infant formulas tested negative for exosomes markers CD63, HSP70 and Alix (Supplemental Figure 2D and Supplemental Figure 3, Supplemental Digital Content 6), and tested positive for bovine caseins for formulas 2 and 3 (Supplemental Figure 2B). Alpha-lactalbumin was not detected in milk formula 1, was abundant in formula 2 and was present in trace amounts in formula 3. Consistent with this observation, formula 1 is prepared by using hydrolyzed whey protein hydrolysate, whereas formulas 2 and 3 list nonfat milk as major ingredient (Supplemental Table 2, Supplemental Digital Content 2). We detected trace amounts of alpha-tubulin (marker for microvesicles), integrin β (found in most classes of EVs) and butyrophilin (marker for fat globules) in vesicles purified from infant formulas and human and bovine milk (Supplemental Figure 2B). Human β-casein was detected in human milk but not in vesicles purified from human milk.

MicroRNA Expression

Two hundred twenty-one mature microRNAs were identified in the three samples of exosomes isolated from fresh human milk; 84 microRNAs were common among samples (Supplemental Figure 4, Supplemental Digital Content 7). The ten most abundant

microRNAs accounted for more than 70% of the total sequencing reads (Table 1). Milk samples stored at 4°C for less than 24 h prior to exosome isolation yielded quantities of small RNAs sufficient for microRNA-sequencing analysis (~ 12 ng/μL), whereas the yield was low if milk was stored at −80°C or for longer than 24 h (< 1 ng/μL).

Column Contamination

No contamination with microRNAs was detected in MinElute columns. All Ct values above the detection limit in RT-qPCR. Ct values of miR-30d-5p were 34.4 ± 0.3 and 34.0 ± 0.2 in non-treated and hypochlorite-treated spin columns, respectively. Ct values of miR-423-5p were 32.5 ± 2.4 and 35.0 ± 1.0 in non-treated and hypochlorite-treated spin columns, respectively. miR-125a-5p was not detectable after 40 cycles in both non-treated and hypochlorite-treated spin columns. The external standard, miSpike produced Ct values close to the detection limit in non-treated (28.5 ± 1.1) and hypochlorite-treated (29.9 ± 0.5) spin columns in RT-qPCR (n = 3, $P > 0.05$).

MicroRNA Analysis in Human Milk, Milk Exosomes and Infant Formulas

RT-qPCR analysis of microRNAs produced expression patterns similar to those observed by sequencing analysis, although the expression of miR-30d-5p and miR-423-5p were near the detection limit of RT-qPCR (Supplemental Figure 5A, Supplemental Digital Content 8). Analysis of exosome-sized vesicles from infant formulas resulted in Ct values above detection limit for miR-30d-5p and miR-423-5p (> 29 cycles) and no signal after 40 cycles for miR-125a-5p. MicroRNAs were detected in whole human milk but not in non-fractionated formulas (Supplemental Figure 5B). Expression was greater in unfractionated milk compared to milk exosomes (Supplemental Figure 5A vs. 5B).

Discussion

This study advances the field of human milk exosomes and their microRNA cargos as follows. First, human milk exosomes and microRNA cargos can be analyzed using small volumes of human milk. Second, storage conditions need to be taken into account before engaging in large-scale studies of exosomes and their cargos in human milk. Third, exosome-sized particles in infant formulas are not exosomes. Fourth, the concentration of microRNAs is below detection limit in infant formulas and in exosome-sized particles isolated from formulas. Fifth, we could not reproduce observations that RNeasy MinElute spin columns are contaminated with microRNAs [14].

To date, only a few studies have explored the phenotypes of milk exosome depletion in infants or suckling animals. Infants fed soy formula experienced a slight delay in cognitive development compared with breastfed infants in a longitudinal study [16]. Assessment of exosome-dependent phenotypes will benefit from the availability of microRNA data collected using protocols that adhered to current standards of sample storage and exosome isolation and authentication. This paper laid the groundwork for future studies of exosomes and their microRNA cargos in large cohorts of women with a high degree of confidence that discoveries will stand the test of time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors acknowledge the use of the Biomedical and Obesity Research Core and the assistance of Dr. Steven Kachman in the Nebraska Center for the Prevention of Obesity Disease through Dietary Molecules (NIH 1P20GM104320) at the University of Nebraska-Lincoln, and the services provided by the DNA Sequencing Core at the University of Nebraska Medical Center (NIH P20GM103427, 1P30GM110768 and P30CA036727). The authors acknowledge the Holland Computing Center at the University of Nebraska-Lincoln for providing computational support.

Source of Funding: JZ receives research support from the National Institute of Food and Agriculture (NIFA), U.S. Department of Agriculture, under award numbers 2015-67017-23181 and NIFA2016-67001-25301, National Institutes of Health (NIH) grant 1P20GM104320, the University of Nebraska Agricultural Research Division (Hatch Act), U.S. Department of Agriculture multistate group W3002, and the University of Nebraska President's Office. JC receives research support from the University of Nebraska President's Office. JZ serves as a consultant for PureTech Health, Inc.

Abbreviations:

bta	Bos taurus
Ct	cycle threshold
DMSO	dimethylsulfoxide
EVs	extracellular vesicles
hsa	Homo sapiens
miR	microRNA
N.D.	not detectable
RT-qPCR	quantitative reverse transcription PCR

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What is Known

- Exosomes and their microRNA cargos from milk are bioavailable.
- Dietary depletion of milk exosomes and their microRNA cargos elicits phenotypes in infants and piglets.
- Exosome preparations may be contaminated with non-exosome particles of similar size.

What is New

- Storage of human milk is a confounder in the analysis of exosomes and their cargos.
- MicroRNA-sequencing analysis suggests that 10 microRNAs account for more than 70% of the 221 mature microRNAs detected in exosomes from human milk.
- Exosome-sized particles in infant formulas are likely casein micelles, and the microRNA content in formulas is below detection limit.
- This paper provides guidelines for the analysis of vesicles and RNA in human milk.

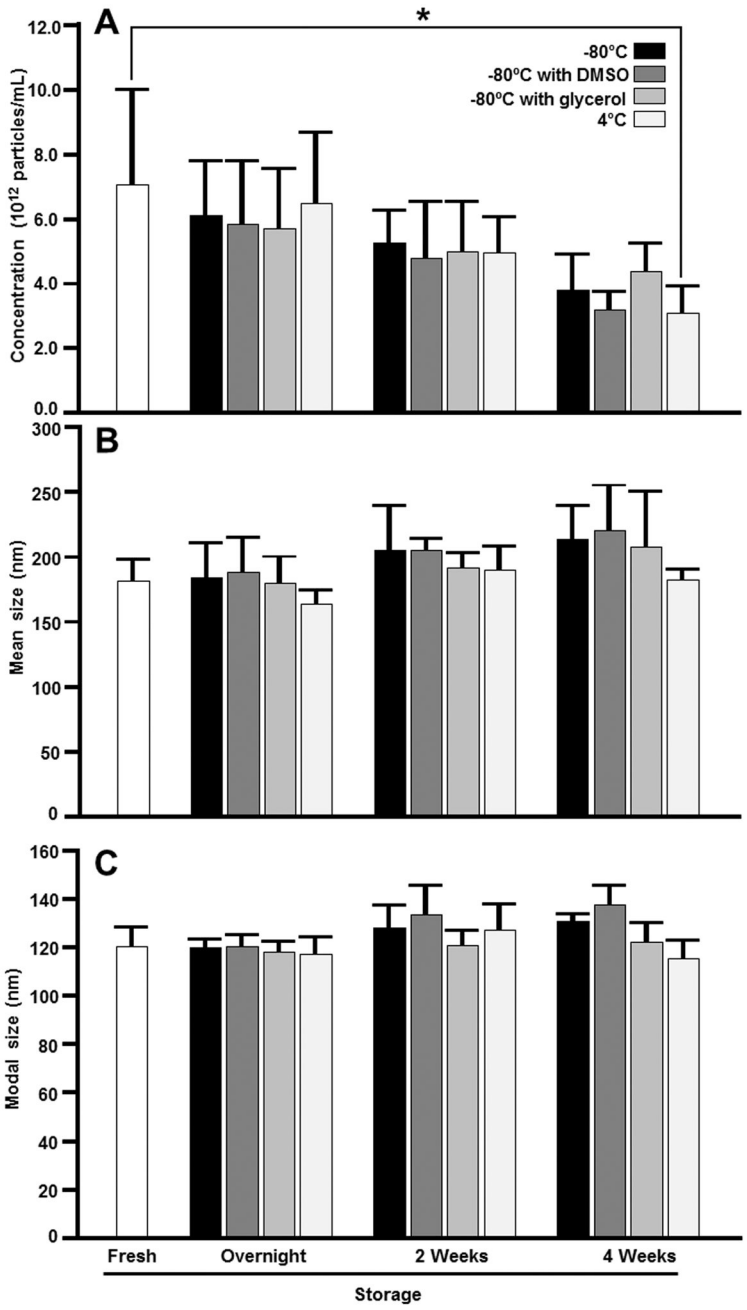

Figure 1.

Concentration (A), mean size (B) and modal size (C) of exosome-sized vesicles in human milk stored at −80°C, −80°C with 5% DMSO, −80°C with 10% glycerol, and 4°C compared with fresh human milk. Data are expressed as means ± SEM (n = 3 biological repeats, **P* = 0.03).

Table 1.

Normalized counts for top ten microRNAs detected in human milk exosomes (n = 3)

microRNA	Normalized Counts[1]	Cumulative %
hsa-miR-30d-5p*	238,233 ± 122,360	29.9%
hsa-let-7b-5p	111,414 ± 47,752	43.9%
hsa-let-7a-5p	72,111 ± 17,294	53.0%
hsa-miR-125a-5p*	24,679 ± 2,485	56.1%
hsa-miR-21-5p	23,222 ± 26,043	59.0%
hsa-miR-423-5p*	20,667 ± 8,638	61.6%
hsa-let-7g-5p	19,890 ± 5,877	64.1%
hsa-let-7f-5p	19,022 ± 951	66.5%
hsa-miR-30a-5p	18,589 ± 16,448	68.8%
hsa-miR-146b-5p	17,246 ± 10,660	71.0%

[1] In expression analysis by miRDeep2, values are normalized by library size and multiplied by a factor of 10^6 which corresponds to counts per million mapped microRNA reads.

* Confirmed by RT-qPCR.