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Breast milk-derived exosomes promote intestinal epithelial cell growth

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ABSTRACT

Background: Breast milk administration prevents necrotizing enterocolitis (NEC). However, the mechanism remains unclear. Exosomes are cell-derived vesicles highly present in human milk and regulate intercellular signaling, inflammation, and immune response. We hypothesized that milk-derived exosomes beneficially affect intestinal epithelial cells.

Methods: Rat milk was collected, and exosomes were isolated using ExoQuick reagent and visualized by Nanoparticle Tracking Analysis. Protein was extracted from encapsulating exosomes, and concentration was measured. 2×10^4 intestinal epithelial cells (IEC-18) were treated for five hours with 0.5-μg/μl exosomes, an equal volume of exosome-free milk, or control solution (PBS). IEC-18 viability was measured using a colorimetric assay (MTT), and gene expression was analyzed by qRT-PCR. Data were compared using one-way ANOVA with Bonferroni post-test.

Results: Rat milk was collected, and exosome isolation was confirmed. Compared to control, treatment with exosomes significantly increased IEC viability, proliferation, and stem cell activity (all $p < 0.05$). However, administration of exosome-free milk had less significant effects.

Conclusions: Rat milk-derived exosomes promote IEC viability, enhance proliferation, and stimulate intestinal stem cell activity. These findings provide insight into the mechanism of action of breast milk in the intestines. Exosome administration is a promising prevention method for infants at risk of developing NEC when breastfeeding is not tolerated.

Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in newborn infants, with an incidence between 0.5 and 5 per 1000 live births, primarily affecting preterm and low birth weight neonates [1]. The condition is characterized by ileal and/or colonic necrosis, ranging in severity from mild intestinal inflammation to intestinal perforation, multisystem organ failure, and death [1]. Despite advances in health research, the mortality rate from NEC remains above 30%, and the pathogenesis of the disease is still incompletely understood, demonstrating the need for continued studies in this field [1].

It is well known that breastfeeding is associated with a decreased incidence of NEC [2]; however, the factors that mediate the ability of breast milk to prevent NEC development remain inconclusive. Exosomes are cell-derived vesicles released by most tissues, and present in the majority of body fluids, including breast milk [3]. They range from 50 to 150 nm in diameter, and mediate functions in intercellular signaling, inflammation, immune response, cell adhesion, waste management, and protection against stress [4]. The vesicles are formed when a multi-vesicular endosome fuses with the plasma membrane, and by exocytosis, releases its intraluminal vesicles as exosomes [4].

Some research exists on the effects of exosomes in NEC. *In vitro* and *in vivo,* exosomes derived from bone marrow-derived mesenchymal stem cells have been found to home to injured intestinal segments and protect the intestines from NEC [5], but to our knowledge, the influence of breast milk-derived exosomes in NEC has not been studied.

Breast milk is known to be a rich source of exosomes, with early milk containing a greater exosome concentration compared to mature milk [3]. Proteins, microRNA and mRNA are encapsulated by exosomes within a phospholipid membrane to protect them from ribonuclease digestion, freeze–thaw cycles and acidity [6]. This stability allows exosome contents to reach the neonate's intestinal lumen intact and be absorbed, in order to exert their effects. Preterm mothers, however, are often unable to provide sufficient breast milk, which results in the use of milk banks [7]. This milk is pasteurized, which is a process that has been shown to disrupt exosomal membranes and degrade contents, decreasing their concentration by approximately 50%, and preventing the

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infants from benefitting from the protective effects of these exosomal contents [8].

The aim of the present study was to determine the effect of breast milk-derived exosomes on intestinal epithelial cells. We hypothesized that exosomes isolated from breast milk have beneficial effects on intestinal epithelial cell viability, proliferation and intestinal stem cell activity. Clinically, we suspected that these nanoparticles present a promising prevention method for infants at risk of developing intestinal disorders such as NEC, particularly when maternal breastfeeding is not tolerated.

1. Methods

1.1. Rat breast milk collection

All animal experiments were approved by the Animal Care Committee of The Hospital for Sick Children (no. 32239), and all methods were performed according to its guidelines and regulations. Breast milk was collected from rats between seven and fourteen days postpartum, as this is the period of highest milk production [9]. Milk was collected from six female rats, in order to accumulate enough milk to isolate exosomes for cell treatment. Prior to collection, the mother was separated from her pups for four hours to allow for milk accumulation. After six hours of separation from pups, there is a down-regulation of milk production resulting from mammary gland involution because of lack of suckling [10]. Milk was collected using a small milking pump (Natsume Seisakusho, Tokyo, Japan) while under anesthesia with isoflurane, following an intraperitoneal injection of 0.2 ml oxytocin (2 IU/ml) [9]. Collected milk was kept on ice until centrifugation.

1.2. Exosomes

1.2.1. Exosome isolation

Shortly after collection, before milk freezing and storage, rat breast milk was centrifuged to eliminate fat, cells and debris [11]. Raw milk was centrifuged at 2000 × *g* for 10 min at 4 °C to remove the upper fat layer, and again at 12000 × *g* for 30 min at 4 °C to eliminate deposited cells and debris. The remaining milk was further centrifuged at 12000 × *g* at 4 °C for an additional 5 min to pellet down any remaining cells. Exosomes were isolated from the remaining milk using ExoQuick reagent (System Biosciences, Palo Alto, CA) [12]. A volume of ExoQuick reagent equal to one fifth of the milk volume was added to the milk, which was gently mixed by inverting and refrigerated at 4 °C overnight, according to the manufacturer's protocol. The exosome pellet was obtained by centrifugation at 18000 × *g* for 45 min at 4 °C, resuspended in PBS and stored at −20 °C until experimental use. After exosome isolation, the remaining exosome-free milk was used for comparison purposes.

1.2.2. Exosome visualization

Particles were visualized by Nanoparticle Tracking Analysis (NanoSight LM10, Malvern Instruments Ltd., UK), which uses light scattering and Brownian motion to measure particle size and concentration, as a method of confirming exosome isolation.

1.2.3. Exosome quantification

To quantify the exosomes present in each sample, protein was extracted from the encapsulating vesicles using a cell extraction buffer (Invitrogen, CA, USA), containing Protease Inhibitor Single-Use Cocktail (Sigma, MO, USA). Protein concentration was determined using Bicinchoninic Acid (BCA) Protein Assay (Thermo Scientific, IL, USA), according to the manufacturer's protocol.

1.3. Treatment of intestinal epithelial cells with exosomes

A rat small intestine epithelial cell line, IEC-18 (ATCC, Manassas, VA), was cultured as previously described [13]. 2×10^4 IECs were seeded per well in a 96 well plate, and treated for five hours with 0.5-μg/μl exosomes, an equal volume of exosome-free milk, or a control solution (PBS).

1.4. Assessing intestinal epithelial cell viability

IEC-18 cell viability was measured using a colorimetric assay that detects the conversion of MTT into formazan (ATCC, Manassas, VA), as described previously [13].

1.5. Gene expression analysis

RNA was isolated from the treated intestinal epithelial cells using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of RNA using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg). SYBR green-based qRT-PCR was carried out with advanced qPCR Supermix (Wisent Inc., Quebec, Canada), with the primers outlined in Table 1.

1.6. Statistical analysis

Results are presented as mean ± SEM, as data are normally distributed. Data were compared using one-way ANOVA with Bonferroni post-test. $P < 0.05$ was considered significant.

2. Results

2.1. Effective milk collection technique

Using our milk collection method (Fig. 1A), we were able to collect approximately 1 to 1.5 ml of milk per rat. This volume was then greatly reduced by the removal of fat, cells and debris, resulting in a remaining milk volume, to which ExoQuick reagent was added, equal to approximately one fifth of the original volume of raw milk collected (Fig. 1B). Centrifugation then resulted in a visible exosome pellet (Fig. 1C).

2.2. Confirmation and characterization of rat milk-derived exosomes

Following identical dilution protocols, protein concentrations were ten times greater in the rat milk-derived exosome sample compared to the exosome-free milk, which were 3360.3 μg/ml and 359.68 μg/ml respectively (Table 2). As previously reported, protein concentration is an effective method of assessing the quantity of exosomes present in the sample [14].

The presence of exosomes in our sample was also confirmed by visualization using Nanoparticle Tracking Analysis, and compared to the exosome-free milk. Mean particle size was 263.7 nm ± 93.4 nm in the exosome sample, compared to 288.3 nm ± 107.7 nm in the exosome-free milk (Fig. 2A). This is not a significant difference in diameter; however, there is a significantly lower concentration of vesicles of this size in the exosome-free milk compared to the exosome sample, with the peaks occurring at 2.5×10^7 particles/ml and 5.0×10^7 particles/ml, respectively (Fig. 2A, B).

Table 1
Primer sequences for qRT-PCR.

Name	Forward sequence	Reverse sequence
PCNA (proliferating cell nuclear antigen)	GCCGAGAT CTCAGCCATATT	ATGTACTTAG AGGTACAAAT
Lgr5 (leucine-rich repeat-containing G-protein coupled receptor 5)	CTTCCAACCT CAGCGTCTTC	TTTCCCGCAA GACGTAACTC
GAPDH (glyceraldehyde 3-phosphate dehydrogenase)	TGAAGCAGG CATCTGAGGG	CGAAGGTGGAA GAGTGGGAG
RPLO (50S ribosomal protein)	CCATCAGCAC CACAGCCTTC	GGCGACCTGG AAGTCCAACT