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Human Breast Milk-Derived Extracellular Vesicles in the Protection Against Experimental Necrotizing Enterocolitis

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Abstract

PURPOSE: Necrotizing enterocolitis (NEC) is a leading cause of death in premature infants. Breast feeding decreases the incidence of NEC but, even with aggressive promotion of nursing in Neonatal Intensive Care Units, morbidity and mortality remain high. Previous studies from our laboratory have demonstrated that extracellular vesicles (EVs) purified from mouse and rat stem cells can protect the intestines from NEC. The aim of this study was to determine whether human breast milk (BM)-derived EVs could prevent NEC.

METHODS: EVs were purified from human donor breast milk. NEC was induced in premature rat pups by exposure to asphyxia/hypothermia/hypercaloric feeds. Pups were randomized to: (1) breast fed, no injury, (2) NEC (3) NEC + BM-derived EVs once intraperitoneally (IP), (4) NEC + BM-derived EVs enterally (PO) with each feed. Intestinal tracts were examined for histologic damage. Additionally, the effect of BM-derived EVs on intestinal epithelial cells (IEC) subjected to hypoxia/reoxygenation injury *in vitro* was examined.

RESULTS: NEC incidence was 0% in breast-fed pups and 62% in pups subjected to NEC. IP administration of BM-derived EVs decreased NEC incidence to 29% and enteral administration further decreased NEC incidence to 11.9%. ($p < 0.05$). BM-derived EVs significantly increased cell proliferation and decreased apoptosis in IEC *in vitro*.

CONCLUSION: Breast milk-derived EVs delivered either IP or enterally significantly decrease the incidence and severity of experimental NEC, protect IEC from injury *in vitro*, and may represent an innovative therapeutic option for NEC in the future.

Keywords

NEC; extracellular vesicle; breast milk; exosome

Necrotizing enterocolitis (NEC) is a devastating disease of newborns, with mortality rates that remain as high as 20–30%. The cost of treating infants with NEC exceeds \$1 billion in

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the US each year (2). Babies who survive NEC frequently have long term complications including short bowel syndrome, developmental delays and neurological sequelae (1–3). Unfortunately, despite much research over the past decades, a cure has not been identified.

Previous research has demonstrated that different types of stem cells (SC) have the ability to reduce the incidence and severity of experimental NEC. Furthermore, extracellular vesicles (EVs) derived from SC are similarly protective against NEC, suggesting that they mediate the therapeutic effects of the SC from which they originate (1, 2). EVs are nanovesicles, ~20–200nm in size, that are released by many cells in the body and are ubiquitously present in body fluids including saliva, urine, and breast milk (4). Whereas prior studies relied on intraperitoneal (IP) administration of EVs for NEC therapy, (2), enteral administration would be more clinically relevant. Recent research has suggested that the enteral route could be a promising way to deliver EVs (5, 6).

Promotion of breast feeding regimens in neonatal intensive care units has decreased the incidence of NEC, although the exact therapeutic components in BM have yet to be fully defined (4–7). Recent research on breast milk-derived EVs has demonstrated their ability to endure gastric and pancreatic digestion (6). This makes them very attractive as a clinical treatment that can be easily administered. Despite growing interest in the biological properties of human breast milk-derived EVs, there are very few published studies regarding their therapeutic applications in NEC. Rat and bovine breast milk-derived EVs have been reported to augment proliferation of intestinal epithelial cells *in vitro* (5, 7). However, the effects of human breast milk-derived EVs *in vitro* or in *in vivo* NEC models are unknown. The goal of the current study was to examine human breast milk-derived EVs, delivered either IP or PO, as a novel prevention strategy in experimental NEC.

1. Methods

1.1. Cell culture

IEC-6 cells (ATCC CRL-1592) derived from rat small intestinal epithelium were grown in 1X Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose, L-glutamine and sodium pyruvate (Corning, Corning, New York), with additional supplementation of 10% fetal bovine serum and 1% penicillin-streptomycin-amphotericin B. FHs 74 int cells (ATCC CCL-241) derived from fetal small intestine were grown in Hybrid-Care Medium (ATCC-46X), with additional supplementation of 15% fetal bovine serum, 1% penicillin-streptomycin-amphotericin B, and 30 ng/mL recombinant human EGF (R&D Systems, Minneapolis, MN). Cells were cultured in a 37°C water-jacketed humidified incubator maintained at 5% CO₂. Both cell types were used for *in vitro* assays upon reaching ~70–80% confluency.

1.2. EV purification and characterization

Breast milk-derived EVs were isolated from human breast milk that had been donated to the Ohio Health Milk Bank (Columbus, OH). EVs were purified using a series of ultracentrifugation steps as follows: (1) 3000g for 15 min, 3 times to remove fat, (2) 10,000g for 30 min with supernatants retained, (3) 100,000g for 70 min, twice, with the pellet

retained and resuspended in phosphate buffered saline (PBS). Quantification of EVs was performed using nanotracking analysis (NTA) on a NanoSight NS300 instrument (NanoSight, Malvern Instruments Ltd., Worcestershire, UK).

1.3. Western Blotting

Purified EVs were resuspended in PBS and lysed on ice for 15 min in 1X cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing a protease inhibitor mixture (Sigma, St. Louis, MO). Lysates were cleared of insoluble material by centrifugation at 16,000g at 4°C for 15 min, and protein concentrations determined using a Bicinchoninic Assay (BCA) protein assay kit (ThermoFisher, Columbus, OH). Samples were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, probed with Flotillin rabbit mAb, Alix mouse mAb (Cell Signaling Technology, Danvers, MA), CD81 mouse mAb (ThermoFisher, Columbus, OH) or CD63 mouse mAb (Abcam, Cambridge, MA). Membranes were blotted with corresponding secondary antibodies (rabbit anti-donkey and mouse anti-rabbit) and detected by Western blotting using ECL reagent (Biomed, Dallas, TX).

1.4. Transmission electron microscopy (TEM)

Transmission electron microscopy was used to confirm the size and morphology of EVs. Formvar/carbon coated mesh grids were glow discharged, exposed to the EV suspension for 2 min, counterstained with 1% aqueous uranyl acetate, and imaged using a Tecnai Spirit transmission electron microscope (Columbus, OH) at 80kV.

1.5. Experimental model of NEC

Animal experiments were ethically conducted under protocol #AR1500012 approved by the Institutional Animal Committee of the Research Institute at Nationwide Children's Hospital. Sprague Dawley rat pups were delivered prematurely via Cesarean section at E21 of the normal 22-day gestation period. Premature pups were randomized to the following groups: (1) breast fed, no injury (n=13); (2) NEC + 50 µL sterile water IP (n=70); (3) NEC + a single dose of 4×10^8 BM-derived EVs in 50 µL IP (n=17) or (4) NEC + 1×10^8 BM-derived EVs PO by inclusion in the formula and administration by gastric gavage with every feed for the duration of the experiment (n= 42). Treatments were initiated shortly after delivery.

Following C-section, breast fed pups were placed with a surrogate dam, were uninjured, and were untreated. All other pups were subjected to repetitive episodes of stress over the next 96 hours to induce experimental NEC. Hypercaloric feeds of Esbilac milk replacer (PetAg, Hampshire, IL) with Similac 60/40 powder (Ross Pediatrics, Columbus, OH) were delivered every 4 hours via gastric gavage. This formula delivered 836.8kJ/kg per day. Feeds were initially started at 0.1 mL of formula and increased by 0.1mL of formula daily, reaching 0.4 mL per feed on day four. Also, the second feed on the first day of the experiment included 2 mg/kg of lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO). Pups were placed in a sealed container of nitrogen gas for 90 sec three times daily to induce hypoxia (oxygen concentration <1.5%). To achieve hypothermia, pups were placed in 4°C for 10 min three times a day. After 96h or upon clinical signs of NEC, pups were sacrificed and intestinal tissue collected and fixed in 10% formalin (Fisher Scientific, Pittsburg, PA).

1.6. Histologic grading

Intestinal specimens were embedded in paraffin, sectioned (5 μ m), and subjected to hematoxylin and eosin (H&E) staining. Samples were graded separately by two blinded reviewers, and mucosal damage graded using an established intestinal injury grading scale as follows: Grade 0, normal mucosa; Grade 1, epithelial cell lifting; Grade 2, necrosis to the midvillus level; Grade 3, necrosis of the entire villus; and Grade 4, transmural mucosal necrosis (8). Any score of Grade 2 or higher was defined as being consistent with experimental NEC.

1.7. XTT Cell Proliferation Assay and H/R Regimen

An XTT Cell Proliferation Assay (American Type Culture Collection, Manassas VA) was performed according to the manufacturer's instructions. Briefly, IEC-6 or FHS-74 int cells were plated at 10,000 cells/well and allowed to adhere overnight. The medium was then replaced for 5h with serum-free medium containing 0 – 1×10^8 BM-derived EVs / 100 μ L. A hypoxia/reoxygenation (H/R) regimen was initiated by switching the cells to pre-reduced hypoxic media containing the same EV concentrations, and placing them in a modular incubator chamber under hypoxic conditions (0.5–1.5% total oxygen) for 48 h. The cells were then switched to normoxic medium to induce reoxygenation for 4 h. Control cells were grown under normoxic conditions for the duration of the experiment.

XTT reagent was added to each well and plates were incubated at 37°C for 2 h. Plates were then read at 450 nm with a reference read at 650 nm on a Molecular Devices SpectraMax M2 (San Jose, CA) plate reader. The percentage of proliferation compared to control was calculated by dividing the normalized OD of the hypoxia groups by the normalized OD of the untreated normoxic control and multiplying by 100. For XTT analysis, a total of four to six biological replicates were used. Each biological replicate consisted of three technical replicates that were averaged together.

1.9. Annexin Apoptosis Assay

Annexin-V expression was analyzed using the Annexin V-FITC Apoptosis Staining/ Detection kit (Abcam, Cambridge, MA) per manufacturer's instruction. Briefly, 100,000 cells were plated in 6-well plates and allowed to adhere overnight. Cells were then pre-treated with 1×10^8 exosomes / 100 μ L of media followed by H/R as described above. Cells were then collected and re-suspended in Annexin-V binding buffer. Cells were labeled with Annexin V-FITC and Propidium Iodide antibodies, and analyzed on a BD LSR flow cytometer (Franklin Lakes, NJ). 10,000 cellular events were collected for each sample. Flow data were analyzed using FlowJo vX.0.6. Late apoptotic cells were defined as Annexin-V+ / Propidium Iodide+ cells, and live cells as Annexin-V- / Propidium Iodide- cells. Data were presented as the percentage of late apoptotic or live cells that comprised the 10,000 events measured, and were averaged from three independent experiments with two replicates per experiment.

1.8. Statistical analyses

All data represent mean $\pm$ SD. Differences in NEC incidence and severity between groups were analyzed using Fisher's exact test and Welch's t-test where appropriate. Statistical

significance for cell proliferation and apoptosis assays was determined using the one-way ANOVA with Tukey's post-hoc analysis. For histologic grade averages, a Kruskal-Wallis test was run, followed by a post-hoc Dunn's multiple comparisons test to evaluate group changes. All data analyses were performed using GraphPad Prism 8. p values <0.05 were considered statistically significant.

2. Results

2.1. EV characterization

Differential ultracentrifugation of breast milk produced samples that were enriched in nanovesicles that were predominantly ~100–200 nm in diameter as assessed by NTA (Figure 1A). Typically, the enrichment procedure yielded $\sim 1.6 \times 10^{12}$ EVs of purified sample from a starting volume of 6 ml of human milk. Western blotting confirmed the presence of the established EV membrane markers CD63, CD81, Alix, and Flotillin (Figure 1B) (9–11). TEM illustrated the EV with the characteristic cup shaped morphology and size of ~100 nm (Figure 1C).

2.2. Histological analysis

The average histologic injury grades for each group of pups is presented in Figure 2, with representative histologic images of that grade shown (Figure 2A–D). As expected, pups exposed to NEC had significant intestinal injury (average injury grade 1.9) compared to breast fed uninjured pups (average injury grade 0, $p < 0.05$). In contrast, pups exposed to NEC that received BM-derived EVs IP displayed significantly less intestinal damage (average injury grade 0.8) compared to untreated NEC pups ($p < 0.05$). Finally, pups exposed to NEC that received BM-derived EVs PO had even less intestinal damage (average injury grade 0.4).

2.3. In vivo NEC incidence and severity

Breast fed uninjured control pups did not have any intestinal injury (Figure 3). In comparison, untreated pups exposed to stress developed histologic injury consistent with NEC 62% of the time ($p < 0.05$). In contrast, pups exposed to NEC that were treated with BM-derived EVs given IP or PO had significantly lower incidences of NEC compared to untreated pups. Those treated with BM-derived EVs (IP) had a 29% incidence of NEC ($n=19$, $p < 0.05$), and those treated with BM-derived EVs (PO) had an 11.9% incidence of NEC ($n=42$, $p < 0.05$).

2.4. In vitro XTT cell proliferation

Treating IEC-6 rat intestinal epithelial cells with 1×10^5 – 1×10^8 human BM derived-EVs significantly rescued the H/R-mediated reduction in cell proliferation compared to untreated cells ($p < 0.05$) (Figure 4A). Likewise, FHs 74 fetal intestinal epithelial cells that received 1×10^7 human BM derived EVs also displayed a significant increase in cellular proliferation after H/R compared to untreated cells ($p < 0.05$) (Figure 4B).

2.5. In vitro Annexin apoptosis

Finally, the effect of BM-derived EVs on apoptosis was examined. Exposure of IEC-6 cells to H/R significantly increased the percentage of late apoptotic cells compared to control cells grown under normoxic conditions ($p < 0.05$), whereas treatment of H/R-exposed cells with 10^8 BM-derived EVs / 100 μ L significantly reduced apoptosis ($p = 0.05$) (Figure 4C). Additionally, exposure of IEC-6 cells to H/R significantly reduced the percentage of live cells compared to normoxic control cells ($p < 0.05$), whereas treatment with of H/R-exposed cells with BM-derived EVs significantly increased the percentage of live cells ($p = 0.05$) (Figure 4D).

3. Discussion

NEC remains the leading cause of death in premature infants despite decades of research. It is well known that breast feeding decreases the incidence of NEC, but even with aggressive promotion of nursing in Neonatal Intensive Care Units, morbidity and mortality remain unacceptably high. Conventional methods of treatment including bowel rest and decompression, antibiotics, and surgery have not significantly changed the outcomes of these babies over several decades, and given the rapidity with which this disease frequently develops, the best treatment for this disease is likely to be prevention.

Although it has been shown that several different types of SC reduce the incidence and severity of experimental NEC, SC research in clinical medicine is difficult and faces many ethical, legal, and scientific challenges, including a theoretical concern for potential tumorigenicity (2). This concern led to a follow-up study, which examined EVs released from SC and their ability to protect the intestines from NEC. This study indicated that SC-derived EVs reduced the incidence and severity of experimental NEC just as effectively as the SC from which they were derived (2). Additionally, mesenchymal SC-derived EVs have been shown to help regulate the immune system in certain diseases, including renal disease (12). They have also demonstrated improvements in traumatic brain injury in rodent models, as their size allows for permeation through the blood brain barrier (13).

EVs are beneficial as therapeutics in that they are immunologically inert and non-toxic. We have shown in the current study that human breast milk-derived EVs significantly decrease the incidence and severity of NEC. EVs have a vital role in cellular communication and many other biological processes because of their ability to deliver components of their molecular payload (comprising DNA, RNA, micro-RNA and proteins) into recipient cells, which can thereby become functionally reprogrammed (8,9). Whereas previous studies showed that enteral administration of bovine or rat breast milk-derived EVs protected the intestines from NEC in *in vitro* and *in vivo* models (5, 7), our study of EVs from human breast milk and their protective role in NEC *in vivo* provides an important advance in the field that has clear clinical implications. Although we have yet to identify the underlying mechanisms involved, new proteomic studies have suggested that proteins derived from BM-EVs may play a role in regulating development of the GI tract (14, 15).

This study demonstrates that EVs derived from human BM are effective in preventing experimental NEC in a well verified rodent model. We have also shown that BM-derived

EVs possess both anti-apoptotic and pro-proliferative effects in intestinal epithelial cells exposed to H/R. As this research is further refined, optimal dose and timing of BM-derived EVs and the identity of their constituent therapeutic agents are important questions for further investigation. Human BM-derived EVs may prove to be a novel therapeutic option to combat clinical NEC in the future.

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How this paper advances the field:

This study demonstrates that extracellular vesicles derived from human breast milk can prevent experimental necrotizing in rodents. Human BM-derived extracellular vesicles may prove to be a novel therapeutic option to combat clinical necrotizing enterocolitis in the future.

Figure 1. Characterization of BM-EVs.

(A) NTA showing size and frequency of EVs present in purified human BM samples. (B) Immunoblots showing the presence of proteins that are commonly found in EVs from various biological systems. (C) TEM of BM-EV.

Figure 2. Intestinal Histology.

Shown are representative images of intestine from the treatment groups shown above. The graph indicates the average histologic grade in that specific group. Decimals have been rounded to the nearest whole integer and that grade was used to show the histologic representations. (A) breastfed, uninjured (normal architecture, Grade 0), (B) NEC (Grade 2) (C) NEC + one dose of 4×10^8 BM-EVs IP, (Grade 1) and (D) NEC + 1×10^8 BM-EVs with each feed PO, (Grade 0). * $p < 0.05$ vs. breast fed. ** $p < 0.05$ vs. NEC. All data represent mean $\pm$ SD.

Figure 3. Effect of EVs on the incidence and severity of NEC.

Pups were treated as indicated, with the IP group receiving a single dose of 4×10^8 EVs /50 μ L PBS and the PO group receiving 1×10^8 EVs per feed in their formula. The bars represent the total incidences of each histologic injury grade. The number above each bar represents the total percentage of NEC in each group. Histologic Injury Grades: Grade 0=normal mucosa, Grade 1=epithelial cell lifting, Grade 2=necrosis to midvillus level, Grade 3=necrosis of the entire villus, Grade 4=transmural mucosal necrosis. Injury grade 2 is consistent with experimental NEC. BM, breast milk. * $p < 0.05$ vs. breast fed. ** $p < 0.05$ vs. NEC.

Figure 4: Proliferation and apoptosis of intestinal epithelial cells *in vitro* in response to human BM-derived EVs.

(A, B) **XTT cell proliferation assay.** Cell lines were treated with the indicated amounts of BM-derived EVs/100μL media for 5 h. Cells were then exposed to hypoxia for 48 h followed by reoxygenation for 4 h, with continuous treatment with EVs for the duration of the assay. The XTT assay was then performed. Compared to non-treated cells, statistically significant proliferation after H/R was observed in (A) rat IEC-6 cells treated with 1×10^5 – 1×10^8 BM-derived EVs or (B) human FHs 74 cells treated with 1×10^7 BM-derived EVs. (C, D) **Annexin V apoptosis assay.** IEC-6 cells were pre-treated with 1×10^8 exosomes / 100 μL of media followed by the H/R regimen and labeled with Annexin V-FITC and Propidium Iodide antibodies. Flow cytometry was then performed. (C) represents late apoptotic cells and (D) represents live cells. * $p < 0.05$. H/R, hypoxia/reoxygenation; NA, no addition. All data represent mean $\pm$ SD.