



A comparison of exosomes derived from different periods breast milk on protecting against intestinal organoid injury

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Abstract

Aim of the study Human breast milk reduces the risk and severity of necrotizing enterocolitis (NEC). Exosomes are extracellular vesicles (EVs) found in high concentrations in milk, and they mediate intercellular communication and immune responses. The aim of this study is to compare the protective effects of exosomes that are derived from different time periods of breast milk production against intestinal injury using an *ex vivo* intestinal organoid model.

Methods Colostrum, transitional and mature breast milk samples from healthy lactating mothers were collected. Exosomes were isolated using serial ultracentrifugation and filtration. Exosomes' presence was confirmed using transmission electron microscopy (TEM) and western blot. To form the intestinal organoids, terminal ileum was harvested from neonatal mice pups at postnatal day 9, crypts were isolated and organoids were cultured in matrigel. Organoids were either cultured with exposure to lipopolysaccharide (LPS), or in treatment groups where both LPS and exosomes were added in the culturing medium. Inflammatory markers and organoids viability were evaluated.

Main results Human milk-derived exosomes were successfully isolated and characterized. LPS administration reduced the size of intestinal organoids, induced inflammation through increasing TNF α and TLR4 expression, and stimulated intestinal regeneration. Colostrum, transitional and mature human milk-derived exosome treatment all prevented inflammatory injury, while exosomes derived from colostrum were most effective at reducing inflammatory cytokine.

Conclusions Human breast milk-derived exosomes were able to protect intestine organoids against epithelial injury induced by LPS. Colostrum exosomes offer the best protective effect among the breast-milk derived exosomes. Human milk exosomes can be protective against the development of intestinal injury such as that seen in NEC.

Keywords Human breast milk · Exosomes · Intestinal organoids · Necrotizing enterocolitis (NEC)

Introduction

Necrotizing enterocolitis (NEC) is an inflammatory intestinal disease mainly found in 5–10% of premature and low weight birth infants [1]. Although considerable advancements in neonatal medicine were made, the mortality rate

of necrotizing enterocolitis remains as high as 50%. With continuous studies on the contributing factors to NEC over the past several decades, pathogenesis of the disease remains poorly understood [2].

The advantages of human breast milk on infants born prematurely include reduction of occurrence of NEC [3]. Human milk is a dynamic body fluid which the bioactive compound composition changes through different lactation episodes. Although it is believed that these components are responsible for the beneficial effects of human breast milk in reducing the risk and severity of NEC, the mechanism of such protection has not yet been elucidated [4, 5].

Exosomes are extracellular vesicles (EVs) (30–150 nm) found in high concentrations in milk [6]. As a major cell–cell communication messenger, exosomes contain proteins, lipids and genetic materials which can be transported among

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cells to mediate intercellular communication and immune responses [7, 8]. Furthermore, exosomes can be isolated from human breast milk, and can be absorbed into intestinal epithelial cells to exert functions *in vitro* [9].

Recent developments in intestinal epithelial cell culturing have enabled a novel, more *in vivo*-like organotypic *ex vivo* model. These organoids resemble original tissue in structure and function [10, 11]. Through mimicking the natural microenvironment of the gut using the *ex vivo* model, the development of intestinal organoids may help to reduce animal experimentations and the rising failure rates in clinical trials [12]. This new approach has already been applied to drug metabolism and pathological studies, including NEC research. LPS administration to intestinal organoids decreased proliferation, while human milk was shown to exert a protective effect through downregulation of *TLR4* (Toll-like receptor 4) [13, 14]. However, the effect of exosomes derived from human milk of different lactation episodes on intestinal organoid inflammation is unknown.

In this study, we sought to simulate intestinal injury using organoid model under conditions that mimic those in the infant gut environment, and to investigate the effect of the interaction of EVs derived from human breast milk with these three-dimensional (3D) injured intestinal organoids. In addition, we also aimed to compare the effects of breast milk-derived exosomes collected from different lactation periods at preventing intestinal organoid injury. We hypothesize that human breast milk exosomes exert protective effects on injured intestinal organoids through reducing inflammatory responses. Furthermore, we hypothesize that the protective effects of exosomes from different periods breast milk on intestinal injury is different.

Materials and methods

Human breast milk collection

Nine lactating mothers who had delivered preterm infants were enrolled between January 2019 and June 2019 at the Children's Hospital, Fudan University. We collected colostrum (days 1–5 postpartum), transitional milk (days 6–14 postpartum) and mature milk (beyond day 15 postpartum) from these mothers (Table 1). All mothers were producing an excess of milk. Additionally, all mothers were over 18 years of age and healthy without any autoimmune conditions.

Exosomes isolation

Colostrum ($n=3$), transitional ($n=3$) and mature ($n=3$) human breast milk was used. Exosomes were isolated from milk by serial centrifugations. All centrifugations were

Table 1 Clinical information for the breast milk collected

	Sample number	Gestational age at delivery	Infant age at milk collection
Colostrum	1	26w2d	3d
	2	27w5d	4d
	3	28w5d	5d
Transitional milk	4	31w4d	7d
	5	30w1d	9d
	6	28w3d	14d
Mature milk	7	33w4d	21d
	8	27w6d	23d
	9	29w1d	23d

Colostrum (days 1–5 postpartum), Transitional milk (days 6–14 postpartum), Mature milk (beyond day 15 postpartum)

performed at 4 °C. Briefly, milk was centrifuged at 2000×g for 10 min to aspirate fat layer. Supernatant was transferred to a new tube, and centrifugation was performed again at 12,000×g for 40 min to eliminate cellular debris and somatic cells. Then, the new supernatant was filtered by 0.22 μm pore PES membrane through vacuum filtration. The exosomes were obtained from the filtered supernatant by ultracentrifugation at 100,000×g for 2 h at 4 °C using a Beckman Coulter L-90 K ultracentrifuge. After removing the exosome-free supernatant, the pellet was resuspended in 500 μL phosphate buffered saline (PBS) in 1.5 mL Eppendorf tube, and then stored at – 80 °C until use for experiment.

Exosomes confirmation

Exosomes were observed and micrograph images were taken by transmission electron microscope (TEM). Place the samples on formvar coated copper plate for 2 min, rinse with ultra-pure water, and dye 1% uranyl acetate with negative staining. Then the samples were observed by JEOL 1010 transmission electron microscope operated at 80-kV, and the images were captured with an Olympus soft-imaging Veleta digital camera.

Exosomes quantification

Protein concentration was quantified using a Bicinchoninic acid (BCA) reagent kit (Sigma Aldrich, Castle Hill, New South Wales, Australia). All 9 exosome samples collected from human breast milk were quantified against a bovine serum albumin (BSA) standard (0–2000 μg/mL) (Sigma Aldrich, Castle Hill, New South Wales, Australia) to determine protein concentration.

Intestinal organoids

An intestinal organoid model was established by isolating the crypts of terminal ileum harvested from neonatal C57BL/6 mice pups at postnatal day 9. Crypts were processed by multiple cycles of centrifugations and flushing, before suspension in 3D matrigel domes together with mouse organoid media (Stem Cell, IntestiCult Organoid Growth Medium). Lipopolysaccharide (LPS, Sigma, L2630) was added to the media to induce organoid epithelial injury, while in the treatment groups, 0.1 $\mu\text{g}/\mu\text{L}$ of exosomes from either colostrum, transitional or mature human breast milk were also added together with the presence of LPS. These organoids were allowed to grow in different conditions for 48 h. Photomicrographs of organoids were taken by using a microscope IX71 with a DP72 camera (Olympus). The parameters (size and number of organoids) of each image were generated by the software Image J (1.52a version). Each value was presented as a mean of the analysis of at least three wells.

Gene expression

RNA was extracted from intestinal organoids using TRIzol (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. RT-qPCR was performed to measure expression levels of inflammatory markers TNF- α , TLR4 and stem cell marker Lgr5 (Leucine-rich repeat-containing G-protein coupled receptor 5) to compare between control organoids, organoids exposed with LPS, and organoids treated with LPS plus exosomes. Results were generated from three independent experiments, each performed in technical triplicate. Expression levels were calculated by the $\Delta\Delta C_t$ method and normalized to the reference house-keeping gene GAPDH.

Immunofluorescence

Immunofluorescence staining for Ki67 was performed to assess proliferation capacity. For cultured organoid immunofluorescence staining, organoids were seeded on cover glass, fixed with 4% paraformaldehyde for 20 min at room temperature, and incubated with 0.5% Triton X 100 for another 20 min at room temperature. After blocking of non-specific binding, samples were incubated with primary antibodies Ki67 overnight at 4 °C, and with fluorescent secondary antibodies for 1 h at room temperature in dark condition. DAPI was added for visualization of cell nuclei. Immunofluorescent images of stained organoids were captured with a laser scanning confocal microscope

Statistical analyses

All data are presented as mean \pm SD. $*p < 0.05$ and compared using one-way ANOVA with Bonferroni post-test.

Results

Exosomes were successfully isolated, confirmed and quantified

According to our primary confirmation through TEM images, exosome isolation was proven to be performed successfully. Bicinchoninic acid (BCA) quantified $5.4 \pm 0.1 \mu\text{g}/\mu\text{L}$ for colostrum milk-derived exosomes, $4.1 \pm 0.5 \mu\text{g}/\mu\text{L}$ for transitional milk-derived exosomes, and $3.8 \pm 0.7 \mu\text{g}/\mu\text{L}$ for mature milk-derived exosomes. Purified breast milk exosomes were used in the following experiments.

Prevention of intestinal injury by administration of human milk-derived exosomes in ex vivo intestinal organoids

Gene expression levels of inflammatory markers TNF- α and TLR4 were significantly higher with LPS administration for 48 h (Fig. 1a, b). Milk exosomes effectively prevented the upregulation of these two genes induced by LPS. Furthermore, there were significant differences between colostrum, transitional and mature milk exosomes treatment at reducing

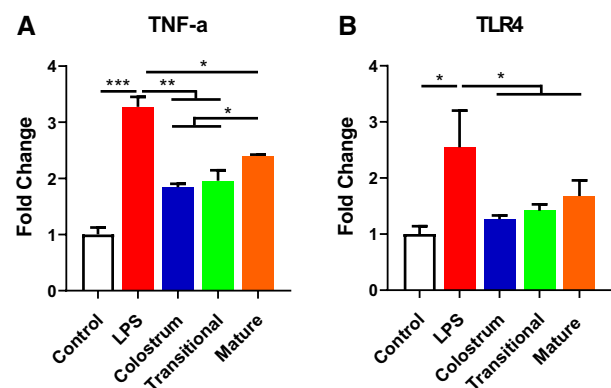


Fig. 1 Exosomes prevented intestinal injury ex vivo. Relative expression of inflammatory markers TNF- α (a) and TLR4 (b) in intestinal organoids treated with LPS alone, LPS with 0.1 $\mu\text{g}/\mu\text{L}$ of exosomes from colostrum, transitional or mature human breast milk, for 48 h. All human breast milk exosome administration prevented upregulation of both TNF- α and TLR4 induced by LPS, where exosomes from colostrum administration significantly decreased expression of TNF- α compared with transitional and mature milk. Experiments were independently repeated three times. Data are presented as mean \pm SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, using one-way ANOVA with post hoc tests

inflammation. Among the three groups, exosomes derived from colostrum milk were most effective.

Exosomes prevented upregulation of intestinal regeneration induced by LPS

To analyze regeneration ability, organoids number showed no difference when comparing between control to LPS group to exosome treatment group (Fig. 2a, b). On the other hand, size of proliferating crypts is associated with the regeneration ability of the crypt cells [15]. In our study, we showed that LPS administration reduced the size of organoids, which means that such injury had a negative impact on intestinal regeneration (Fig. 2a, c). However, when exosomes were administered together with LPS, the size reduction was avoided (Fig. 2a, c).

The expression of Lgr5, a marker of intestinal stem cell [15], revealed that LPS stimulated intestinal regeneration, possibly as a repair mechanism in response to LPS induced injury (Fig. 3c). However, when LPS was administrated with the presence of exosomes, the expression of Lgr5 remained low, similar to the level found in control group. Whereas there were no differences observed between exosomes derived from colostrum, transitional, and mature milk.

The results of immunofluorescence staining indicated that the proliferation marker Ki67 protein expression was

increased in the LPS-induced injured organoids (Fig. 3a, b). We suspect that this again is a repair mechanism which the intestinal cells were trying to increase proliferation to replace the injured cells. And when LPS is administered with the presence of exosomes, Ki67 expression was shown to be lower than the injured group. However, there was no differences between exosomes derived from colostrum, transitional and, mature milk in suppressing the upregulation of Ki67 by LPS. Thus, human breast milk derived-exosomes treatments significantly hampered the injury induced by LPS administration.

Discussion

Studies have reported that exosomes can be isolated from human milk efficiently and can be absorbed by intestinal epithelial cells to exert functions [9]. In this study, we extended our investigations by mimicking the natural microenvironment of the gut through the development of the ex vivo organoid model, as well as to evaluate the effects of the interaction of human breast milk derived-exosomes with these organoids. On the other hand, bioactive compounds composition, including exosome concentration, of human breast milk varies during different lactation periods. Early milk, known as colostrum, contains a higher concentration

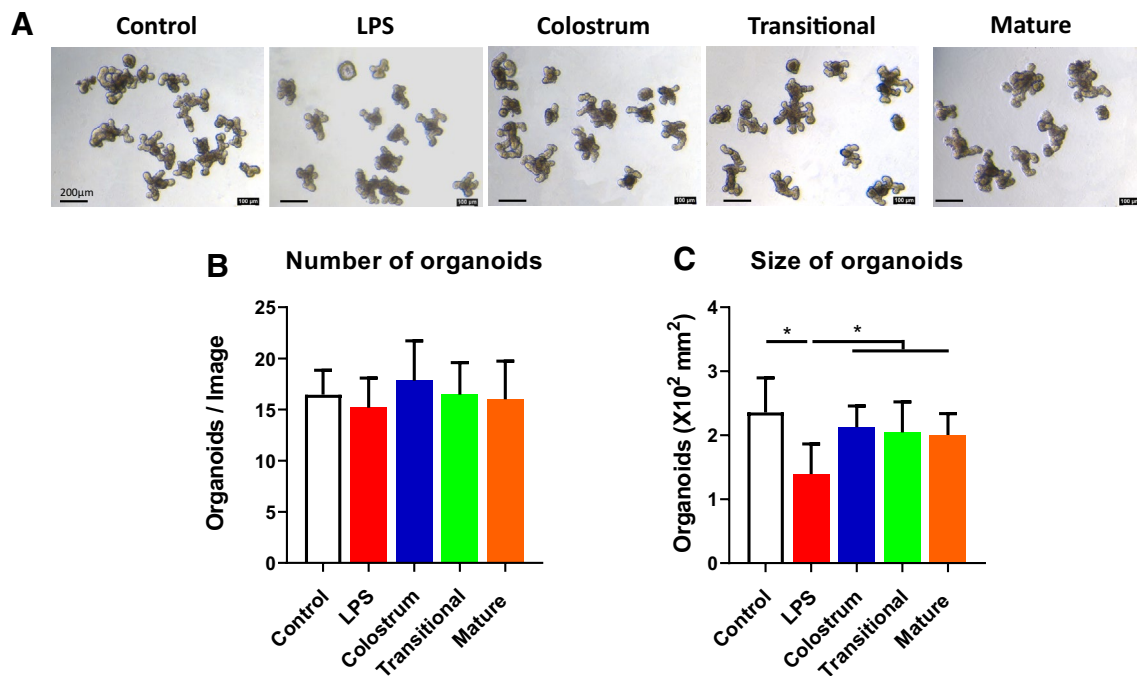


Fig. 2 Exosomes treatment prevented the change in morphology of organoids induced by LPS. Photomicrographs of organoids (a). There was no difference in response to LPS and exosomes in organoids number (b). LPS administration significantly decreased the size of

organoids, and was prevented with exosome administration (c). Each value was presented as a mean of the tests of at least three wells. Data are presented as mean \pm SD. * $p < 0.05$, using one-way ANOVA with post hoc tests

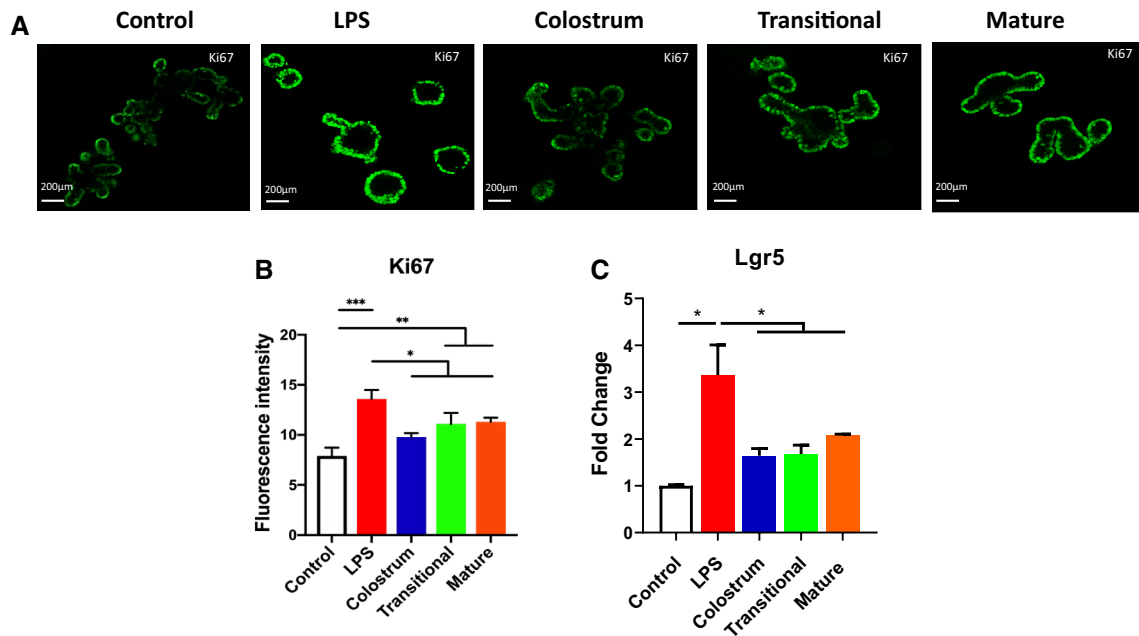


Fig. 3 Exosomes hampered the upregulation of regeneration in injured intestinal organoids. Representative micrographs for Ki67 staining and corresponding quantification of fluorescence intensity in control, LPS, and exosome plus LPS treated organoids (a, b). Relative gene expression of intestinal stem cell marker Lgr5 in each experimental group (c). All human breast milk exosome administra-

tion prevented the upregulation of Ki67 and Lgr5 induced by LPS, whereas there were no differences between exosomes derived from colostrum, transitional and, mature milk. Experiments were independently repeated three times. Data are presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, using one-way ANOVA with post hoc tests

of exosomes than mature milk. Interestingly, the onset of NEC occurs at 2–3 weeks of age, which might be when the mothers stop producing colostrum. Furthermore, it has been showed that administration of exosomes derived from milk collected from mothers that had delivered preterm infants significantly enhance proliferation and migration of intestinal epithelial cells compared to those of full term birth [16]. Thus, in this study, we isolated milk derived-exosomes from different lactation periods collected from mothers who delivered preterm infants.

It has been well established that inflammation in the intestine helps neonates defend against the invasion of pathogens under normal circumstances. However, the intestinal and immune system of NEC neonates are immature, which results in harmful excessive inflammation. In the present study, we were able to prove that human milk-derived exosomes treatment was able to hamper pro-inflammatory responses caused by LPS-induced injury, especially for exosomes derived from colostrum milk. Previous studies reported that the main functional component of milk exosomes is through the microRNAs found within these vesicles, and that the levels of different microRNAs varies among exosomes derived from different periods of milk [17, 18]. Thus, we suspect that the different effects between colostrum, transitional and mature milk on intestinal injury is due to their variation of microRNAs contained in

exosomes. Besides, exosomes treatment significantly influenced TLR4 gene expression in the organoids. It has been reported that human breast milk was able to protect against NEC by inhibition of TLR4 through the epidermal growth factor receptor (EGFR) signaling pathway [19]. Further assessment of the effects of exosomes on the EGFR pathway can lead to the study of the mechanism of protection seen in our current study.

The intestinal regeneration capacity is crucial for repairing epithelium viability and integrity when intestinal injury was induced. Hock et al. reported that rat milk derived-exosomes promoted intestinal cell viability, enhanced proliferation, and stimulated intestinal stem cell activity [9]. Conversely, our study showed that exosomes administration together with LPS prevented the upregulation of proliferation and stem cell activity induced by LPS alone. We speculate that these differences can be explained by the varying injury severity. Previous study showed the capacity of regeneration is up-regulated in intestinal cells with minor injury, and is down-regulated with irreversible injury [20]. When a one stress factor alone, or when stress is given at low concentration, intestinal autonomous repair can be induced by upregulating regeneration mechanism. In our study, the protection by exosomes on intestinal organoids was able to prevent the activation of such autonomous repair. On the other hand, when high level of damage is induced, which

might even disrupt or inhibit intestinal self-repair, we suspect that the effects by exosome as reported by Hock et al. will still be effective in rescuing the damaged epithelium, rather than protection as seen in our study. Thus, an organoid model with administration of more than one stress factors should be developed to aid in future studies focusing on using exosomes as a rescue treatment for NEC.

Exosomes isolated from human breast milk, especially colostrum, can be protective against the development of intestinal injury such as that seen in NEC. The onset of NEC occurs at 2–3 weeks of age when maternal colostrum is no longer produced. Given that this is the population most affected by NEC, prolonged administration of exosomes rich colostrum from milk bank present a possible preventive strategy against development of NEC in preterm infants.

Conclusion

This study contributes toward our understanding of the importance of exosomes as a major functional component in human breast milk. The observations provide us with insights on possible mechanisms underlying the role of breast milk in preventing severe intestinal diseases such as necrotizing enterocolitis. These results showed supporting evidences of using breast milk-derived exosomes as a promising therapy or nutrition strategy for such challenging disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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