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Protective Effects of Human Milk-Derived Exosomes on Intestinal Stem Cells Damaged by Oxidative Stress

Ping Dong[1], Ying Zhang[1], Dong-yong Yan[1], Yi Wang[1], Xiu Xu[1], Ying-chun Zhao[2], and Tian-tian Xiao[2]

Abstract

Breastfeeding has been shown to have a protective effect on the occurrence of necrotizing enterocolitis (NEC), but the mechanism remains unclear. In the context of NEC pathogenesis, many of the protective properties of exosomes on the intestinal epithelial compartment make it an ideal therapeutic target. In the present study, our hypothesis was that intestinal stem cells (ISCs) would be protected from injury by human milk-derived exosomes (HMDEs). Human breast milk was collected, and exosomes were isolated using ExoQuick reagent. Magnetic-activated cell sorting isolation of prominin-1^+ ISCs was performed from small intestines of neonatal rat. ISCs were treated with or without H_2O_2, and HMDEs, an equal volume of HMDE-free milk, or a control solution [phosphate-buffered solution (PBS)] was added, respectively. In the absence of HMDEs, exposure of ISCs to H_2O_2 led to decreased cell viability. However, addition of HMDEs to ISCs exposed to H_2O_2 led to significantly increased ISC viability. There was a significant upregulation of mRNA expression of Axin2, c-Myc, and Cyclin D1 genes of the Wnt/β-catenin axis in ISCs treated with HMDEs (6.99 ± 2.34, 4.21 ± 1.68, 6.17 ± 2.22, respectively, $P < 0.05$ for all), as compared to control. In the presence of carnosic acid (a specific Wnt/β-catenin signaling inhibitor), the cell viability was significantly decreased. Thus, HMDEs protect ISCs from oxidative stress injury *in vitro*, which were possibly mediated via the Wnt/β-catenin signaling pathway. Our findings indicate that oral administration of HMDEs might be a promising measure in treating NEC or in preventing the development of NEC in high-risk infants when breast milk is not available.

Keywords

human milk, exosomes, cell viability, intestinal stem cells

Introduction

The human gastrointestinal system is covered with a single epithelial cell layer, which is one of the main defense mechanisms for the host to restrict pathogenic bacteria to the intestinal cavity, while ensuring the normal absorption of nutrients through the intestine. When the intestinal barrier is impaired, microorganisms and endotoxins in the intestine can break through the barrier, enter the blood and cause the translocation of bacteria and endotoxins, promote the occurrence of enteric infections, and even develop systemic inflammatory response syndrome. A large number of studies have shown that the occurrence, development, and prognosis of many clinical diseases are related to intestinal barrier damage. Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in newborns, with an incidence rate of 0.5 to 5 per 1,000 live births, and mainly affects preterm and low-birth-weight infants[1]. Although NEC-related research has made great progress in recent years, the mortality rate of NEC is still higher than 30%, and the pathogenesis of the disease is still not fully understood. Disruption of intestinal mucosal barrier function may be an early event in the development of NEC[2].

Breastfeeding has been shown to have a protective effect on the occurrence of NEC[3], but the mechanism is still not well understood. Exosomes are cell-derived vesicles released by most tissues, and present in the majority of body

[1] Department of Child Healthcare, Children's Hospital of Fudan University, Shanghai, China
[2] Department of Neonatology, Children's Hospital of Shanghai, China

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Corresponding Author:
Ping Dong, Department of Child Healthcare, Children's Hospital of Fudan University, Shanghai 200433, China.
Email: dongpingbreeze@163.com

fluids, including breast milk[4]. They range from 50 to 150 nm in diameter, and mediate functions in intercellular signaling, immune response, cell adhesion, inflammation, protection against stress, and so on[5].

There have been some studies on the role 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[6], and rat milk-derived exosomes promote intestinal epithelial cells' viability and proliferation[7]. In a recent study published by Liao et al.[8], they isolated exosomes in the milk of lactating mothers and found that these exosomes can survive *in vitro* digestion and successfully be ingested by intestinal epithelial cells (IECs), identifying 288 mature microRNAs in intestinal epithelium. Besides, human milk-derived exosomes (HMDEs) allow IECs to be protected from oxidative stress, but the mechanism is still not clear[9].

Preterm mothers are often unable to provide sufficient breast milk for their children, which results in the use of milk banks[10]. 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 infants from benefitting from the protective effects of these exosomal contents[11]. Exosomes are naturally enriched in the milk of all lactating women. When breastfeeding is not tolerated or the infant needs to be fed with a pasteurized donor milk or formula for various reasons, the use of HMDEs in the prevention and control of preterm related diseases, including NEC, will be a safe, economical, and promising intervention method.

The intestine is responsible for digestion, absorption, endocrine, and defense functions, and the proliferation and differentiation of intestinal stem cells (ISCs) at the bottom of the crypt are the major cytological basis for intestinal mucosal renewal. Stress such as intestinal ischemia can damage the intestinal epithelial cell lineage, especially stem cells, thereby disrupting normal homeostasis and intestinal barrier function. Between 4 and 6 stem cells at each crypt base generate epithelial progenitor cells in the transit-amplifying zone, which subsequently differentiate and maintain intestinal homeostasis[12]. They have been identified using special markers such as leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) and prominin-1/CD133, in addition to classic +4 long retention cell characteristics[13,14]. Stem cells in some organs, including the intestines, have been shown to respond to stress and to promote recovery from injury[15].

To date, no studies have looked at HMDEs to determine if they have a protective effect on the ISCs. In the present study, our hypothesis was that ISCs would be protected from oxidative stress by HMDEs. The possible mechanism will also be explored. This study provides theoretical and laboratory basis for the better use of breast milk exosomes in the prevention and treatment of NEC and other intestinal diseases in children, especially small preterm infants.

Materials and Methods

Human Breast Milk Collection

Breast milk was collected from lactating mothers who took their children to our healthcare clinic for a routine physical examination between December 2018 and January 2019. All mothers were producing an excess of milk, and were over 18 years of age, reportedly healthy, and without any autoimmune conditions. Written informed consent was obtained from the lactating mothers. The study was approved by the Ethics Committee of the Children's Hospital of Fudan University (Children's Hospital of Fudan University Ethics Protocol 2019–087).

Exosome Isolation and Purification

Exosomes were isolated by differential centrifugation of human milk aliquots (10 ml). Low-speed centrifugation at 2,000 × *g* for 10 min at 4°C was used to remove the fat globule layer. The defatted milk was transferred to a new tube containing RNase inhibitor, followed by centrifugation at 12,000 × *g* for 30 min at 4°C to remove the top fat layer and cellular debris. The supernatant was filtered through an RNase-free syringe equipped with a 0.45-μm pore size polyvinylidene difluoride (PVDF) sterile filter (Fisher Scientific, Pittsburgh, PA, USA) to further eliminate cells and cellular debris. The filtered supernatant was incubated with a 5:1 v/v ratio of milk: ExoQuick-TC solution (SBI system Biosciences, Mountain View, CA, USA) for 12 h at 4°C. This mixed solution was used for subsequent analysis, according to the manufacturer's instruction with minor modifications for milk. Resuspended milk exosomes were stored at −80°C when appropriate for further experiments.

Electromagnetic Imaging

A negative staining technique was employed to visualize the exosomes. An enriched exosome suspension was resuspended in filtered PBS, dispensed on carbon-coated electron microscopy grids on parafilm and left to absorb for 1 min at rtp, then transferred to a drop of Uranyless® solution for 1 min and left to air dry. Excess stain was blotted away. Imaging was performed using JEM1400plus Transmission Electron Microscope at 100 kV (JEOL, Japan).

Exosome Visualization

A nanoparticle tracking analysis system (NanoSight LM10, Malvern Instruments Ltd., UK) was used to determine particle size and particle concentration per milliliter.

Western Blot

Exosome lysate was resuspended in 1.5× Laemmli buffer, subsequently incubated at 95°C for 5 min and centrifuged at 13,000 × *g* for 5 min. Samples were separated on a Novex 4% to 12% Bis-Tris Plus Gel (Life Technologies, Carlsbad, CA,

USA), and transferred onto a PVDF membrane (Millipore, Bedford, Mass, USA). The membrane was activated in 100% methanol and rinsed with double-distilled H_2O (ddH_2O) before transfer. After transfer, membranes were processed for Ponceau red staining. Primary Ab (CD81 and clathrin) was diluted in OBB containing 0.1% Tween-20, and incubated overnight at 4°C. Membranes were then washed 4× (5 min each) with TBS containing 0.1% Tween-20 (TBST) at room temperature. Biotin anti-rabbit or anti-mouse secondary Ab (Invitrogen, Carlsbad, CA, USA), along with streptavidin HRP (Life Technologies, Carlsbad, CA, USA) at a 1:1,000 dilution of each in TBST, was incubated for 1 h at room temperature on a rotator. Membranes were scanned and analyzed using an OdysseyH IR scanner using OdysseyH imaging software 3.0.

Isolation of Prominin-1^+ ISCs

Magnetic-activated cell sorting (MACS) isolation of prominin-1^+ ISCs was performed with reference to a previously described method[16,17]. We excised small intestines from 10 to 12 neonatal rat pups at 3 to 5 days of age. Intestines were opened longitudinally, washed with cold PBS, and cut into 5-mm pieces. Tissue fragments were incubated in 2 mM EDTA/PBS for 30 min on ice. Intervillous epithelia were enriched and centrifuged at 300 × *g* for 5 min and then dissociated by incubation in PBS supplemented with trypsin (10 mg/ml) and DNase (0.8 U/μl) for 30 min at 37°C[18]. Single cells were centrifuged at 300 × *g* for 10 min at 4°C, then resuspended in minimum essential medium, and filtered through 40-μm cell strainers. Strained cells were washed with 10 ml of cold PBS and centrifuged at another 300 × *g* for 10 min at 4°C. The isolation of prominin-1-positive stem cells was done according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA, USA) and run through MACS preseparation filters. MACS separation columns were placed in a magnetic multistand and rinsed with 2 ml PBS/bovine serum albumin (BSA)/ EDTA buffer. Filtered cell suspensions were applied to the columns, the columns were washed two times with 2 ml PBS/BSA/EDTA buffer, and flow-throughs collected as controls. The retained prominin-1^+ cells were harvested by removing the column from the magnetic multistand and eluted the cells into collection tubes using 2 ml PBS/BSA/EDTA buffer. To monitor the purification efficiency, portions of run-throughs and retained cells were centrifuged at 300 × *g* at 4°C and fixed in methanol/acetone (v:v = 1:1) for 30 min. Prominin-1^+ stem cells were maintained in the high-glucose Dulbecco's modified eagle medium with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 10 μg/ml insulin, and 100 μg/ml streptomycin) in an incubator at 37°C with 5% CO_2 until oxidative stress experiments were carried out.

Exposure of Prominin-1^+ ISCs to Oxidative Stress

MACS-isolated prominin-1^+ cells were seeded at a density of 3 × 106 and plated on six-well tissue culture dishes. Cells were treated with or without 200 mM doses of H_2O_2, and 0.5 μg/μl of HMDEs, an equal volume of HMDE-free milk, or a control solution (PBS) was added, respectively.

Table 1. Primer Sequences for Quantitative Reverse Transcriptase Polymerase Chain Reaction.

Genes	Primers (5′→3′)
LGR5	P1: CTTCCAACCTCAGCGTCTTC
	P2: TTTCCCGCAA GACGTAACTC
Hes1	P1: AAGAGGCGAAGGGCAAGAATAA
	P2: GGTGCTTCACAGTCATTTCCAGA
Dll1	P1: TGTGACGAGTGTATCCGCTATCCA
	P2: AGGGCTTATGGTGTGTGCAGTAGT
Dll4	P1: ACTGCGAGAAGAAAGTGGACAGGT
	P2: ACATGAGCCCATTCTCCAGGTCAT
AXIN-2	P1: TGTGAGATCCACGGAAACAG
	P2: CTGCGATGCATCTCTCTCTG
c-Myc	P1: TCCTGTACCTCGTCCGATTC
	P2: GGTTTGCCTCTTCTCCACAG
Cyclin D1	P1: TTGACTGCCGAGAAGTTGTG
	P2: AGGGTGGGTTGGAAATGAAC

P1: sense primer; P2: antisense primer.

Assessing Stem Cell Viability

Stem cell viability was evaluated using the Cyquant cell proliferation assay kit (Invitrogen, Eugene, OR, USA), normalized to the viability of the MACS-isolated prominin-1^+ ISCs in the absence of H_2O_2 and added with PBS, which was designated as 100%.

Gene Expression Analysis

To investigate the mRNA levels of genes regulating ISCs growth and proliferation (LGR5, Hes1, Dll1, Dll4, Axin2, c-Myc, and Cyclin D1), RNA was isolated from the treated ISCs using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of RNA using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg). SYBR Green dye and Mastercycler ep realplex4 (Eppendorf, Germany) were used for real-time PCR with the primers outlined in Table 1.

Statistics

Data are expressed as means and SD. One-way analysis of variance was applied to examine group differences, with further multiple comparisons using a Bonferroni test (Stata ver. 7.0, Stata Corp., USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

Confirmation and Characterization of HMDEs

We collected 10 to 15 ml of human milk per person. This volume was then greatly reduced by the removal of fat, cells,

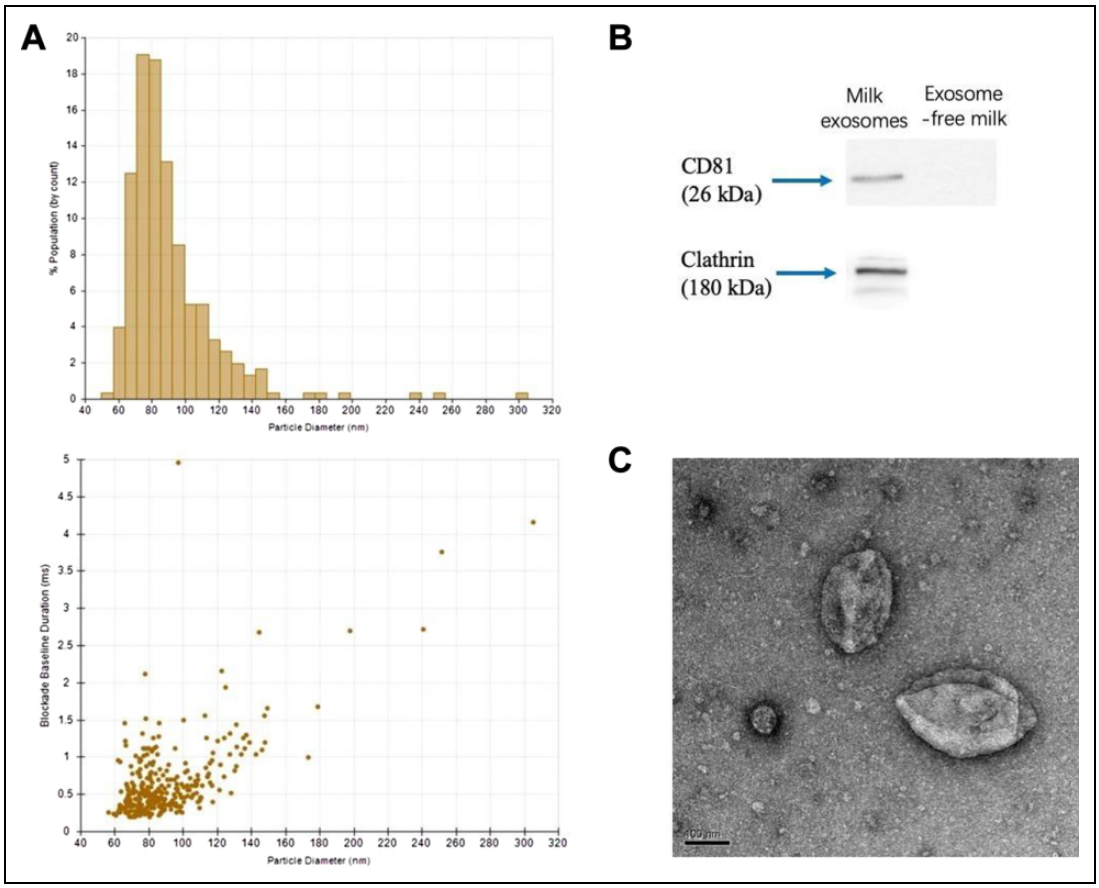

Fig. 1. Characteristics of human breast milk-derived exosomes. (A) Average size (nm) of exosome particles expressed as % population and blockade baseline duration (ms) by nanoparticle tracking analysis. (B) Immunoblot bands demonstrating the presence of CD81 and clathrin at approximately 26 and 180 kDA, respectively. (C) Transmission electron microscopy images of isolated exosomes with negative staining by Uranyless. Circular morphology and the absence of internal staining indicate intact, compartmentalized vesicles.

and debris, resulting in a remaining milk volume, to which ExoQuick-TC solution was added, equal to approximately one-fifth to one-sixth of the original volume of raw milk collected. The exosome pellet in our sample was clearly visible after the centrifugation. The presence of exosomes was further confirmed by visualization using Nanoparticle Tracking Analysis. Particles were in the range of 5.0 to 8.0 $\times$ 10^7 particles per ml; mean particle size was 90.0 $\pm$ 27.7 nm (Figure 1).

The Effect of HMDEs on Prominin-1$^+$ ISCs from Oxidative Stress In Vitro

In the absence of HMDEs, exposure of ISCs to H_2O_2 led to decreased cell viability. However, addition of HMDEs to ISCs exposed to H_2O_2 led to significantly increased ISC viability. Furthermore, under normal conditions, addition of HMDEs also led to increased ISCs viability. HMDE-free milk did not enhance the viability (Figure 2).

HMDEs enhanced ISCs proliferation, as demonstrated by a significant increase in the relative gene expression of Lgr5 following exosome administration (6.33 $\pm$ 3.01, $P < 0.05$; Figure 3A). This effect was not observed in cells treated with HMDE-free milk (2.07 $\pm$ 0.99), compared to control (1.00 $\pm$ 0.85; Figure 3A).

The Possible Mechanism by Which HMDEs Act on Prominin-1$^+$ ISCs

To elucidate the mechanism by which HMDEs act in promoting ISCs growth, we investigated the relative mRNA expression of Axin2, c-Myc, and Cyclin D1genes of the Wnt/β-catenin axis and notch pathway genes (Hes1, Dll1, Dll4). In comparison to control, there was a significant upregulation of Axin2, c-Myc, and Cyclin D1 expression in ISCs treated with HMDEs (6.99 $\pm$ 2.34, 4.21 $\pm$ 1.68, and 6.17 $\pm$ 2.22, respectively, $P < 0.05$ for all). Axin2 gene expression was also increased in cells administered HMDE-free milk (4.14 $\pm$ 1.19, $P < 0.05$), but this increase was of smaller magnitude compared to HMDE-treated cells (Figure 3B), but there were no significant group differences in the expression of Hes1, Dll1, and Dll4 ($P > 0.05$ for all) (Figure 3C).

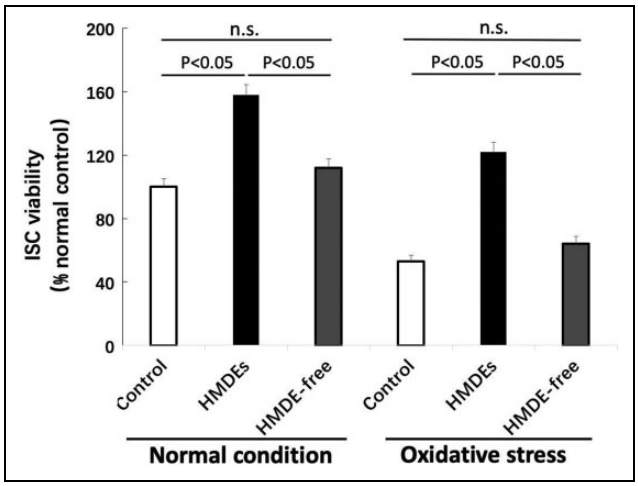

Fig. 2. Human milk-derived exosomes enhance cell viability. ISC viability in either normal or oxidative stress condition was compared in control, exosome, and exosome-free milk-treated cells. Results are mean ± SEM. $P < 0.05$ was considered significant. HMDE: human milk-derived exosome; ISC: intestinal stem cell.

To test this further, we investigated the cell viability, in the presence of HMDEs, with or without carnosic acid (CA, a specific Wnt/β-catenin signaling inhibitor), upon exposure to H_2O_2 or under normal conditions. In the presence of CA, cell viability was significantly decreased. The viability of ISCs cultured in the presence of HMDEs and CA was similar to that of the ISCs without HMDEs. These findings were similar under either normal or oxidative stress conditions (Figure 4).

Discussion

Our study demonstrates for the first time that human breast milk-derived exosomes significantly promote ISCs proliferation and viability. These results highlight the importance of these cell-derived vesicles in breast milk.

A number of exosome purification methods have been developed with adaptation to the biological fluid from which the vesicles are derived. But so far, isolation and purification processes optimized for high yields at minimal time and cost are still lacking in terms of breast milk-derived exosomes. For instance, the storage conditions of milk have been shown to be an important factor for the final exosome integrity and concentration[19]. The most widely used isolation method is differential centrifugation, which selectively removes extracellular debris. However, this method usually produces lower exosomal yields, excess protein is still present, and exosomal integrity is questionable. In our current study, we explored an effective method for collecting milk from lactating mothers and extracting exosomes from this source. As previously described that exosomes range in diameter from roughly[5] 50 to 150 nm, we confirmed the exosome isolation by using Nanoparticle Tracking Analysis. Our results demonstrated a greater concentration of particles in the exosome sample compared to the exosome-free milk sample and the vast majority of particles in our exosome sample falls in this range, and only a few fall above this range. We speculated that the oversized particles could be the exosome agglomerates, which result in larger-diameter recordings.

In the context of NEC pathogenesis, many of the protective properties of exosomes on the intestinal epithelial compartment make it an ideal therapeutic target. Exosome-mediated delivery of epigenetic modifications have been found to have an impact on barrier function[20], pathogenic microbial luminal sensing[21], and the upregulation of antimicrobial peptides in intestinal crypts[22]. All of these factors have been implicated in NEC pathogenesis, including oxidative stress[23]. Martin et al.[9] recently found that HMDEs allow IECs to be protected from oxidative stress, but the mechanism is still not clear. For the first time, we have shown that human breast milk-derived exosomes are a potential therapy to decrease cell toxicity directly in ISCs. The epithelium of small intestine and colon displays a remarkable self-renewal rate, likely necessitated by the constant barrage from physical, chemical, and biological insult. Indeed, the small intestinal epithelium of the mouse completely renews every 3 to 5 days. The intense proliferation that fuels this self-renewal process is confined to the crypts. Individual crypts comprise around 250 cells and generate a similar number of new cells each day. Resident ISCs have long been suspected to be located close to the crypt base[24]. These stem cells produce vigorously proliferating progenitors called transit-amplifying cells, which move upward as coherent columns toward the crypt/villus border. Their migration continues toward the villus tip, where they die and are shed into the lumen. In the intestine, there are active Lgr5-ISCs and relatively stationary label-retaining +4 stem cells. Under physiological conditions, Lgr5-ISCs divide in a symmetrical or asymmetrical manner to maintain the stability of the number of cells in the crypt, while the +4 cells are relatively stationary. However, when the intestinal tract is exposed to acute damage such as radiation, Lgr5-ISCs in crypt columnar cells rapidly die, but +4 cells can be transformed into active Lgr5-ISCs, which continue to differentiate to maintain the survival of intestinal epithelial crypts[25]. In the present study, we used H_2O_2 to induce oxidative stress on ISCs directly. The focus in our study has been to better understand the protective mechanisms of oxidative stress-induced cellular damage and the protective role of breast milk-derived exosomes is proved to be promising.

ISCs are critical for damage-induced intestinal regeneration, but the mechanisms regulating ISC function and inducing epithelial regeneration after tissue damage remain poorly understood. Several signaling pathways including the Wnt/β-catenin and Notch cascades are critical to ISC self-renewal and proliferation[26,27]. Among them, Wnt/β-catenin is the signature signaling pathway, and its downstream regulated genes represent potential ISC markers. The Wnt/β-catenin target gene LGR5 has been recently identified as a

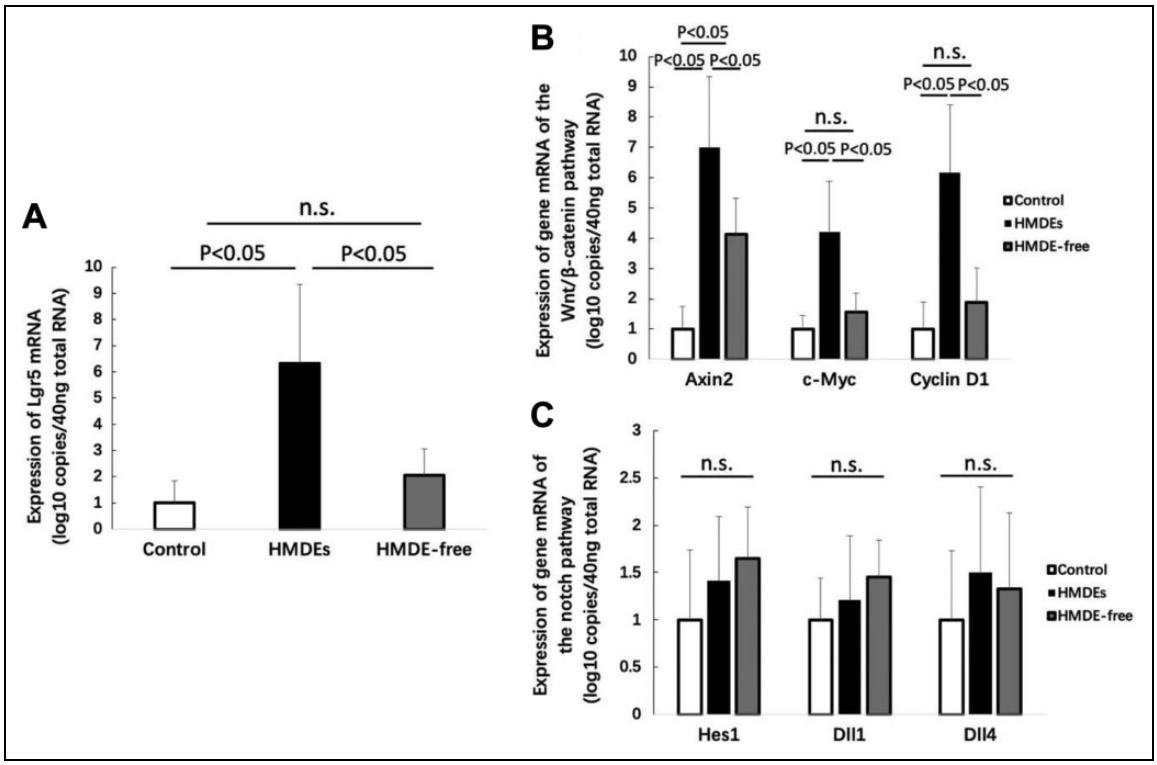

Fig. 3. Changes of related gene expression levels in the ISCs under oxidative stress with or without exosomes supplementation. (A) The expression of gene mRNA of Lgr5, a marker of intestinal stem cell; (B) the expression of gene mRNA of the Wnt/β-catenin signaling pathway (Axin2, c-Myc, and Cyclin D1); (C) the expression of gene mRNA of the notch pathway (Hes1, Dll1, and Dll4). Results are mean ± SEM. $P < 0.05$ was considered significant.
HMDE: human milk-derived exosome; ISC: intestinal stem cell.

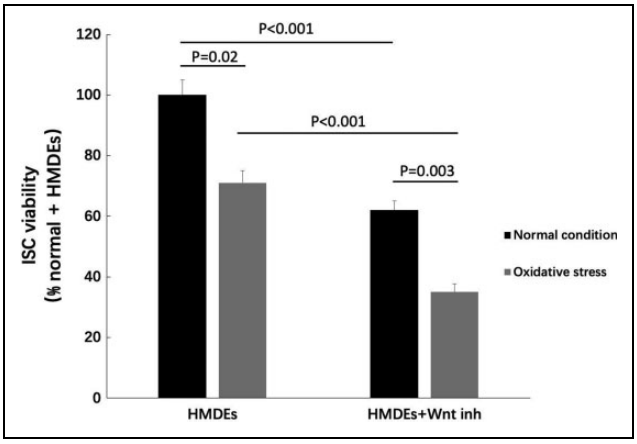

Fig. 4. The enhancement of exosome on cell viability could be reversed by Wnt/β-catenin signaling inhibitor. ISC viability in normal and oxidative stress condition was compared in the presence of HMDEs, with or without carnosic acid (a specific Wnt/β-catenin signaling inhibitor). Results are mean ± SEM. $P < 0.05$ was considered significant.
HMDE: human milk-derived exosome; ISC: intestinal stem cell; Wnt inh: Wnt/β-catenin signaling inhibitor.

marker for ISCs. Here, we found an evidence of enhanced expression of Axin2, c-Myc, and Cyclin D1 genes in the Wnt/β-catenin pathway within ISCs cultured with HMDEs. In addition, we found no HMDEs-induced activation of gene expression in the Notch pathway, which is also critical for ISC maintenance.

In summary, the current studies show that HMDEs protect ISCs from oxidative stress injury *in vitro*, which were possibly mediated via the Wnt/β-catenin signaling pathway. Our findings indicate that oral administration of HMDEs might be a promising therapeutic option in treating NEC. This research also highlights the potential novel application of HMDEs in preventing the development of NEC in high-risk infants when breast milk is not available. Future studies should examine the ability of exogenously administered HMDEs and ISCs, delivered alone and in combination, to protect the intestines from injury due to experimental NEC in vivo.

Ethical Approval

Written informed consent was obtained from the lactating mothers. All experimental procedures involving animals were approved by the Animal Ethics Committee of Shanghai Medical Collage of Fudan University and the care of animals was in accordance with institution guidelines. The study was approved by the Ethics Committee of the Children's Hospital of Fudan University (Children's Hospital of Fudan University Ethics Protocol 2019–087).

Statement of Human and Animal Rights

Written informed consent was obtained from the lactating mothers. All experimental procedures involving animals were approved by the Animal Ethics Committee of Shanghai Medical Collage of Fudan University and the care of animals was in accordance with institution guidelines. The study was approved by the Ethics Committee of the Children's Hospital of Fudan University (Children's Hospital of Fudan University Ethics Protocol 2019–087).

Statement of Informed Consent

Written informed consent was obtained from the lactating mothers who provided their milk for our study.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iD

Ping Dong https://orcid.org/0000-0003-2348-1452

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