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ABOUT COVER

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AIMS AND SCOPE

The primary aim of *World Journal of Stem Cells* (*WJSC*, *World J Stem Cells*) is to provide scholars and readers from various fields of stem cells with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJSC* publishes articles reporting research results obtained in the field of stem cell biology and regenerative medicine, related to the wide range of stem cells including embryonic stem cells, germline stem cells, tissue-specific stem cells, adult stem cells, mesenchymal stromal cells, induced pluripotent stem cells, embryonal carcinoma stem cells, hemangioblasts, lymphoid progenitor cells, *etc*.

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ORIGINAL ARTICLE

Basic Study

Umbilical cord mesenchymal stem cell exosomes alleviate necrotizing enterocolitis in neonatal mice by regulating intestinal epithelial cells autophagy

Lin Zhu, Lu He, Wu Duan, Bo Yang, Ning Li

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Abstract

BACKGROUND

Necrotizing enterocolitis (NEC) is a severe gastrointestinal disease that affects premature infants. Although mounting evidence supports the therapeutic effect of exosomes on NEC, the underlying mechanisms remain unclear.

AIM

To investigate the mechanisms underlying the regulation of inflammatory response and intestinal barrier function by umbilical cord mesenchymal stem cell (UCMSCs) exosomes, as well as their potential in alleviating NEC in neonatal mice.

METHODS

NEC was induced in 5-d-old C57BL/6 pups through hypoxia and gavage feeding of formula containing lipopolysaccharide (LPS), after which the mice received human UCMSC exosomes (hUCMSC-exos). The control mice were allowed to

breastfeed with their dams. Ileal tissues were collected from the mice and analyzed by histopathology and immunoblotting. Colon tissues were collected from NEC neonates and analyzed by immunofluorescence. Molecular biology and cell culture approaches were employed to study the related mechanisms in intestinal epithelial cells.

RESULTS

We found that autophagy is overactivated in intestinal epithelial cells during NEC, resulting in reduced expression of tight junction proteins and an increased inflammatory response. The ability of hUCMSC-exos to ameliorate NEC in a mouse model was dependent on decreased intestinal autophagy. We also showed that hUCMSC-exos alleviate the inflammatory response and increase migration ability in intestinal epithelial cells induced by LPS.

CONCLUSION

These results contribute to a better understanding of the protective mechanisms of hUCMSC-exos against NEC and provide a new theoretical and experimental foundation for NEC treatment. These findings also enhance our understanding of the role of the autophagy mechanism in NEC, offering potential avenues for identifying new therapeutic targets.

Key Words: Necrotizing enterocolitis; Autophagy; Umbilical cord mesenchymal stem cell; Exosomes; Intestinal epithelial cell; Intestinal barrier function

Core Tip: Based on observations of clinical samples, this study revealed a new mechanism by which human umbilical cord mesenchymal stem cells-derived exosomes reduce the inflammatory response and intestinal barrier dysfunction in neonatal mice with necrotizing enterocolitis (NEC) from the perspective of intestinal epithelial cell autophagy. These findings offer new insights for the clinical application of exosomes in NEC treatment and contribute to the establishment of a solid theoretical foundation.

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INTRODUCTION

Necrotizing enterocolitis (NEC) is a gastrointestinal disorder that predominantly affects newborns, particularly premature infants. The incidence rate is approximately 3%, but it increases to 13% among newborns with a birth weight less than 2500 g. The mortality rate can reach 20%-30%[1]. At present, the primary approach for NEC diagnosis and treatment involves nonspecific supportive therapy, which is based on surgical intervention[2]. Nonetheless, the timing of the surgery and the complications that arise following the procedure continue to present clinical challenges. Therefore, it is critical to conduct an in-depth examination of the molecular mechanisms contributing to the development and onset of NEC. This analysis will aid in the identification of more precise and efficient targets for potential treatment strategies, thereby offering the essential theoretical foundation for subsequent translational research bridging the gap between basic scientific knowledge and clinical applications.

Extensive research has shown substantiated that NEC perturbs the equilibrium of proinflammatory and anti-inflammatory signals within the organism, precipitating an intensified succession of inflammatory reactions. This heightened inflammatory reaction exacerbates NEC and contributes to multiple organ failure. Moreover, this reaction adversely affects the clinical prognosis and induces exacerbates harm to the intestinal epithelial barrier[3]. Hence, a severe inflammatory response and impaired functionality of the intestinal mucosal epithelial barrier are two pivotal elements in the development of NEC.

Autophagy exerts varied influences on the gastrointestinal mucosal barrier. Investigative studies have shown elucidated that autophagy upholds the equilibrium of the intestinal mucosal milieu *via* its connections to the intestinal epithelium[4]. Malfunctions in the autophagy signalling pathway may induce harm to Paneth cells within the intestines and diminish the mucosa's capacity to counteract pathogens[5]. Furthermore, autophagy has both inflammatory and anti-inflammatory effects. Under normal circumstances, autophagy governs the secretion of inflammatory cytokines and maintains cellular homeostasis. Nonetheless, excessive autophagy activation during times of stress can trigger the activation of inflammasomes, excessive cytokine secretion, and increased inflammation[6]. Suboptimal maturation of the intestinal mucosal barrier and weakened resistance of the intestinal epithelium to pathogens in premature infants are critical risk factors for the onset of NEC[7]. The influence of autophagy on the intestinal mucosa and inflammatory factors is dual-faceted, indicating its substantial regulatory role in the initiation of disease.

In recent years, stem cell therapy has shown promising results in treating various diseases, including gastrointestinal diseases. Significant progress has been made in this field of animal research. Stem cells have been found to possess several beneficial effects such as anti-inflammatory, anti-apoptotic, and intestinal barrier-enhancing effects. These effects have potential clinical implications for NEC, thus providing a protective effect. A meta-analysis of nine animal experiments demonstrated that stem cells and stem cell-derived exosomes can reduce the incidence of NEC, particularly stage II NEC, by improving intestinal motility and reducing intestinal permeability. Umbilical cord mesenchymal stem cells (UCMSCs) are widely used due to their easy accessibility and noninvasive isolation method. These cells possess various differentiation capabilities and have shown success in animal models of ischemia reperfusion and NEC[8]. However, the specific mechanism of their action is still unclear. Notably, exosomes, which are bioactive factors secreted by stem cells, have shown comparable, or even superior, therapeutic effects. Therefore, the aim of our study was to investigate the mechanisms underlying the regulation of the inflammatory response and intestinal barrier function by UCMSC-derived exosomes, as well as their potential for alleviating NEC in neonatal mice.

MATERIALS AND METHODS

Colon tissue samples from NEC neonates

We collected colon tissue samples from neonates with NEC and used the colon tissue removed during the first operation (the colon tissue was separated 3-5 mm from the edge of the obviously damaged or necrotic site) as the experimental group (NEC). When the stoma was selectively closed, the functional end tissue of the colon served as a control group (Ctr). The present study was approved by the ethical standards of Qilu Hospital of Shandong University ethics committee. This study was performed in accordance with the International Ethical Guidelines for Human Biomedical Research (2012). Information regarding the patients with NEC was provided by the guardians of the patients. Written informed consent was obtained from the participants involved in the study.

Human UCMSC and IEC-18 culture

Human UCMSCs (hUCMSCs) purchased from Cyagen Biosciences (Guangzhou, China) were cultured in MSC medium (OriCell®, Cyagen Biosciences, Guangzhou, China) at 37 °C in a 5% CO_2 incubator. hUCMSCs from passages 3 and 6 were used throughout the experiments.

IEC-18 represent rat ileal epithelial cells. The cells were routinely cultured in DMEM with high glucose containing 10% foetal bovine serum (HyClone) and 1% penicillin/streptomycin (Gibco) in a humidified incubator under 5% CO_2 at 37 °C. IEC-18 cells were exposed to lipopolysaccharide (LPS, 50 μg/mL, Sigma) for 12 h to induce epithelial injury *in vitro*. hUCMSCs exosomes (hUCMSC-exos) (50 μL) were added separately to relieve the injury of IEC-18 cells. Rapamycin (30 nM) were added to clarify the role of autophagy in the above phenomenon.

Exosome extraction and identification

Exosomes were extracted using an exosome extraction kit (ExoQuick-TC, SBI, United States) according to the manufacturer's instructions[9]. The protein content of the exosome suspension was determined using a BCA quantitation kit (Beyotime, Shanghai, China). hUCMSC-exos were used for experiments or stored at -80 °C. The morphology, size, and marker (CD63 and CD81) expression of hUCMSC-exos were analyzed using conventional transmission electron microscopy (TEM), NanoSight, and western blotting, respectively.

Construction and evaluation of the NEC mouse model

C57BL/6J mice were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). Five- to nine-day-old neonatal pups were used to stablish an experimental NEC model according to a previous study[10]. Experimental NEC mice was induced in mice *via* combined 'artificial feeding + hypoxia + LPS' stimulation. Newborn mice underwent the following procedures: (1) Artificial feeding: Formula milk was administered through a gastric tube inserted into the mouth. Artificial feeding was performed every 8 h, with a dose of 40 μL/g of mouse body weight; (2) Hypoxia: After each feeding, the mice were placed in an experimental animal hypoxia chamber for 0.5 h, where they received hypoxia treatment (95% N_2 + 5% O_2 for 10 min); and (3) LPS: On the 2nd and 3rd d of modelling, LPS (4 μg/g of mouse body weight) was added to the formula milk during the second artificial feeding at 6 d (D6) and 7 d (D7) after birth. The modelling process lasted for a total of 96 h and ended on the 9th day of age (D9). All surviving mice were anesthetized with an intraperitoneal injection of pentobarbital (3%, 1 μL/g of mouse body weight) and underwent cardiac puncture. Blood samples were collected, and intestinal samples were obtained by opening the abdominal cavity. The exclusion criteria were as follows: Mice that died due to asphyxia caused by errors in gastric tube insertion during artificial feeding and mice that died within 24 h of the start of modelling. NEC mice were divided into 2 groups: (1) The NEC group: No treatment group; and (2) The Exo group: During the NEC induction process, D6 and D7 mice were intraperitoneally injected with 100 μL of exo.

All procedures involving animals used in this study were performed and monitored in accordance with the guidelines of the Chinese Council on Animal Care and were approved by the Institutional Animal Care and Use Committee of the Qilu Hospital of Shandong University. Throughout the study, all efforts were made to minimize any suffering of the animals.

Hematoxylin-eosin staining

Fixation of the distal ileum tissues was carried out for one night using a solution of 4% paraformaldehyde. These tissues were subsequently embedded in paraffin and sliced into 3 μm thick sections. Afterwards, hematoxylin and 3% eosin were used to individually stain the prepared sections. The histopathological state of the terminal ileum tissues in each group of young animals was meticulously examined and documented *via* the capture of accompanying photographic evidence.

Immunohistochemical and immunofluorescence staining

After deparaffinization and rehydration, the tissue sections were placed in a repair box filled with antigen retrieval buffer containing citric acid (pH = 6.0) and heated in a microwave oven to facilitate antigen retrieval. Subsequently, the slides were transferred to a solution for antigen retrieval and incubated with a primary antibody overnight at 4 °C. The primary antibodies used were against ZO-1 (Cat# GB111402-100, Servicebio) and LC3 (Cat#ab192890, Abcam). After overnight incubation, a secondary antibody was applied at room temperature for 1 h. For immunohistochemical staining, 3,3-diaminobenzidine (Sigma, St. Louis, MO) was used. Counterstaining with haematoxylin was performed, and the sections were examined under an optical microscope. Alternatively, for immunofluorescence staining, Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:400; Servicebio, China) or Cy3-conjugated goat anti-rabbit antibody (1:400; Servicebio, China) was utilized. The cell nuclei were labelled using DAPI solution (SouthernBiotech). The slides were visualized using a fluorescence microscope and the results were quantified using Image-Pro Plus 6.0 software. The expression levels of LC3 and ZO-1 were quantified based on the mean density of 5 randomly selected fields from each group.

Adenovirus infection and laser confocal detection

Ad-mRFP-GFP-LC3 (synthesized by Obio Technology, Shanghai, China)[11] was used to monitor the induction of autophagy and autophagic flux by detecting the degradation of GFP signals under acidic conditions in the lysosome lumen. The experiment commenced by introducing cells into 12-well culture plates at a concentration of 1×10^5 cells/ml in DMEM. Once the cells reached 30% confluence, Ad-mRFP-GFP-LC3 was added to each well at a multiplicity of infection of 50, following the manufacturer's instructions. All treatment procedures were conducted 48 hours after infection. To observe the cells, they were washed three times with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde for 30 min. Finally, confocal microscopy (Olympus, FV3000) was used to capture images of the cells.

Western blot

For immunoblot analysis, mouse ileal tissues and IEC-18 cells were lysed using a total protein extraction kit according to the manufacturer's instructions. The IEC-18 cells were divided into the Ctr, LPS (50 μg/mL, 12 h), LPS + Exo (1 mL culture medium + 50 μL hUCMSC-exos, 24 h), Rap (30 nM, cells pretreated for 1 h), Rap + LPS, and Rap + LPS + Exo. Total lysates were resolved by electrophoresis using 4%-15% precast polyacrylamide gels, transferred to polyvinylidene fluoride membranes, and incubated overnight at 4 °C with antibodies against p62 (Cat# ab109012, Abcam), LC3 (Cat# ab192890, Abcam), ZO-1 (ZO-1, Cat# GB111402-100, Servicebio), Caudin1 (Cat# ab211737, Abcam), E-Cadherin (Cat# ab231303, Abcam), Ki67 (Cat# ab16667, Abcam,) and GAPDH (Cat# AB-P-R001, Goodhere Biotech). The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-mouse IgG-HRP, Cat# SA00001-1, Proteintech; goat anti-rabbit IgG-HRP, Cat# A0208, Beyotime) for 1 h at room temperature and detected using an enhanced chemiluminescence substrate. The analysis of immunoblot images was performed using Bio-Rad's Image Lab software.

TEM

TEM (HITACHI, HT7700, Chiyoda, Tokyo, Japan) was employed to uncover the morphological changes in autophagic structures throughout the dynamic maturation process, spanning from the phagophore to the autolysosome. Cells in each group were collected by cell scrapers and then fixed in 2.5% glutaraldehyde following centrifugation at 800 rpm for 5 min. Subsequently, a postfixation step was performed at room temperature for 2 h using 1% osmium tetroxide (OsO4) in 0.1 M PBS. The sequential procedures of dehydration, infiltration, embedment, and sectioning of ultrathin slices were then carried out. Finally, the ultrathin sections were treated with uranyl acetate and lead citrate for 15 min each, before being observed and imaged using TEM.

Enzyme-linked immunosorbent assay for cytokines

According to the manufacturer's protocol, the levels of interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)-α in mouse serum and the levels of secreted IL-6, IL-8 and TNF-α in IEC-18 cell culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit (mouse IL-6 ELISA kit, Cat# CSB-E04639m; rat IL-6 ELISA kit, CSB-E04640r; mouse TNF-α ELISA kit, Cat# CSB-E04741m; rat TNF-α ELISA kit, CSB-E11987r; all purchased from CUSABIO, Wuhan, China. Mouse IL-8 ELISA kit, Cat# SEKM-0046 and rat IL-8 ELISA kit, Cat# SEKR-0014 purchased from Suolaibao (Beijing, China). Three times ELISA analyzes were repeated three times for each sample. A Multiskan FC photometer (Thermo Scientific) was used to detect the absorbance of each sample at 450 nm.

Migration assays

Cell migration was evaluated through the use of a scratch assay. IEC-18 cells were seeded in 6-well plates at a density of 1.2×10^5 cells/mL and cultivated until they reached complete confluence. To establish a baseline value, five parallel scratches were created in each well using a 200 μL pipette tip, and the width of each scratch was determined. Subse-

quently, the cells were cultured with various substances in a 5% CO_2 incubator for 0 or 12 h. By employing a light microscope from Leica® (Germany), the width of the scratch was observed and quantified through the use of ImageJ software, resembling an exclusion zone assay.

RESULTS

Isolation and identification of hUCMSCs-exos

To investigate the potential roles of hUCMSC-exos in neonatal NEC, hUCMSC-exos were first isolated and verified *via* TEM, western blotting, and nanoparticle tracking analysis (NTA). The NTA data indicated that the diameters of the hUCMSC-exos were mostly approximately 100 nm (Figure 1A). TEM revealed that hUCMSC-exos exhibited a round morphology (Figure 1B), which is consistent with the typical exosomal morphology. Western blot results indicated that the levels of specific exosome surface markers (CD81 and CD63) were significantly increased in the hUCMSC-exos (Figure 1C). Collectively, these results confirmed that the hUCMSC-exos were successfully isolated and identified.

hUCMSC-exos alleviate the inflammatory response and intestinal damage in neonatal mice with NEC

To demonstrate the role of intestinal epithelial cell autophagy in the development of NEC, we conducted a study using colon tissue samples obtained from neonates with NEC. The intestinal villi were seriously damaged in the NEC tissue sections (Figure 1D and E). Immunofluorescence analysis revealed decreased expression of the tight junction protein ZO-1 (Figure 1D and E) and increased expression of the autophagy protein LC3 (Figure 1F and G) in the intestinal epithelial cells of NEC neonates, demonstrating that autophagy may be involved in the development of NEC.

Similar to the finding in NEC neonates, blunting of villous tips and submucosal separation/oedema were observed, and the expression of ZO-1 and LC3 was altered in NEC mice (Figure 1H and I). The concentrations of IL-6, IL-8 and TNF-α were significantly increased in the NEC group (Figure 1J). However, the intraperitoneal administration of hUCMSC-exos during the generation of mouse model of NEC resulted in a significant reduction in intestinal injury severity and cytokines levels and prevented the decreases in ZO-1 and claudin-1 expression compared with those in NEC controls (Figure 1H and I), supporting the protective role of hUCMSC-exos in a mouse of NEC model. We found that the expression of the autophagy markers LC3 and p62 was reversed in the hUCMSC-exos treated group (Figure 1K). Next, we investigated whether the beneficial effects of hUCMSC-exos were associated with enterocyte autophagy.

hUCMSC-exos reduce LPS-induced autophagy in enterocytes

LPS is a commonly used factor in *in vitro* experiments to simulate intestinal epithelial cell damage. The stimulation of IEC-18 cells by LPS notably enhanced autophagy, as indicated by the elevated expression of the autophagy marker protein LC3-II and decreased expression of p62 (Figure 2A). Further examination *via* confocal microscopy revealed a significant increase in the presence of autophagosomes and autophagic lysosomes (Figure 2B). TEM also confirmed a significant increase in the number of autophagic vacuoles (Figure 2C). Additionally, an inverse expression pattern was observed in the hUCMSC-exos- treated group, demonstrating that hUCMSC-exos inhibit the autophagy of enterocyte induced by LPS. Furthermore, after the mechanistic target of rapamycin (mTOR) inhibitor rapamycin activated autophagy, the effects of hUCMSC-exos were abolished, indicating that hUCMSC-exos may regulate autophagy in intestinal epithelial cells through the mTOR signalling pathway. However, whether the regulation of enterocyte autophagy by hUCMSC-exos is involved in its protective effect on NEC is unclear.

hUCMSC-exos reduce LPS-induced inflammation and damage by regulating autophagy and enhancing the migration of intestinal epithelial cells

As shown in Figure 3, hUCMSC-exos enhanced the expression of tight junction proteins (E-cadherin, claudin-1, and ZO-1) and the cell proliferation protein Ki67, which was inhibited by LPS, in IEC-18 cells. They also improved the migration ability of IEC-18 cells and reduced the inflammatory reaction caused by LPS. However, when autophagy is activated by Rap, the effects of the hUCMSC-exos mentioned above are nullified. This indicates that autophagy plays a significant role in the protective effect of hUCMSC-exos in intestinal epithelial cells.

DISCUSSION

In the present study, we observed overactivated autophagy in neonates and mice with NEC, which is in line with earlier studies[12,13]. Additionally, we have provided evidence showing the ability of hUCMSC-exos to mitigate inflammatory responses and intestinal damage in NEC mice and to enhance the migratory capacity of intestinal epithelial cells *in vitro*. Specifically, we found that hUCMSC-exos ameliorate NEC in a manner dependent on enterocyte autophagy. These findings illuminate the underlying mechanism through which hUCMSC-exos confer protection against NEC, which involves the inhibition of excessive intestinal epithelial cell autophagy.

NEC still represents a devastating disease for preterm neonates, and limited preventive and therapeutic strategies are currently available. In recent years, stem cell therapy has emerged as a promising approach for treating various diseases, including gastrointestinal diseases. Animal research has made significant progress in understanding the potential benefits of stem cells in treating NEC[14]. UCMSCs are extensively employed in investigations owing to their convenient accessibility and noninvasive isolation technique. These cells exhibit diverse abilities to differentiate and have been effectively

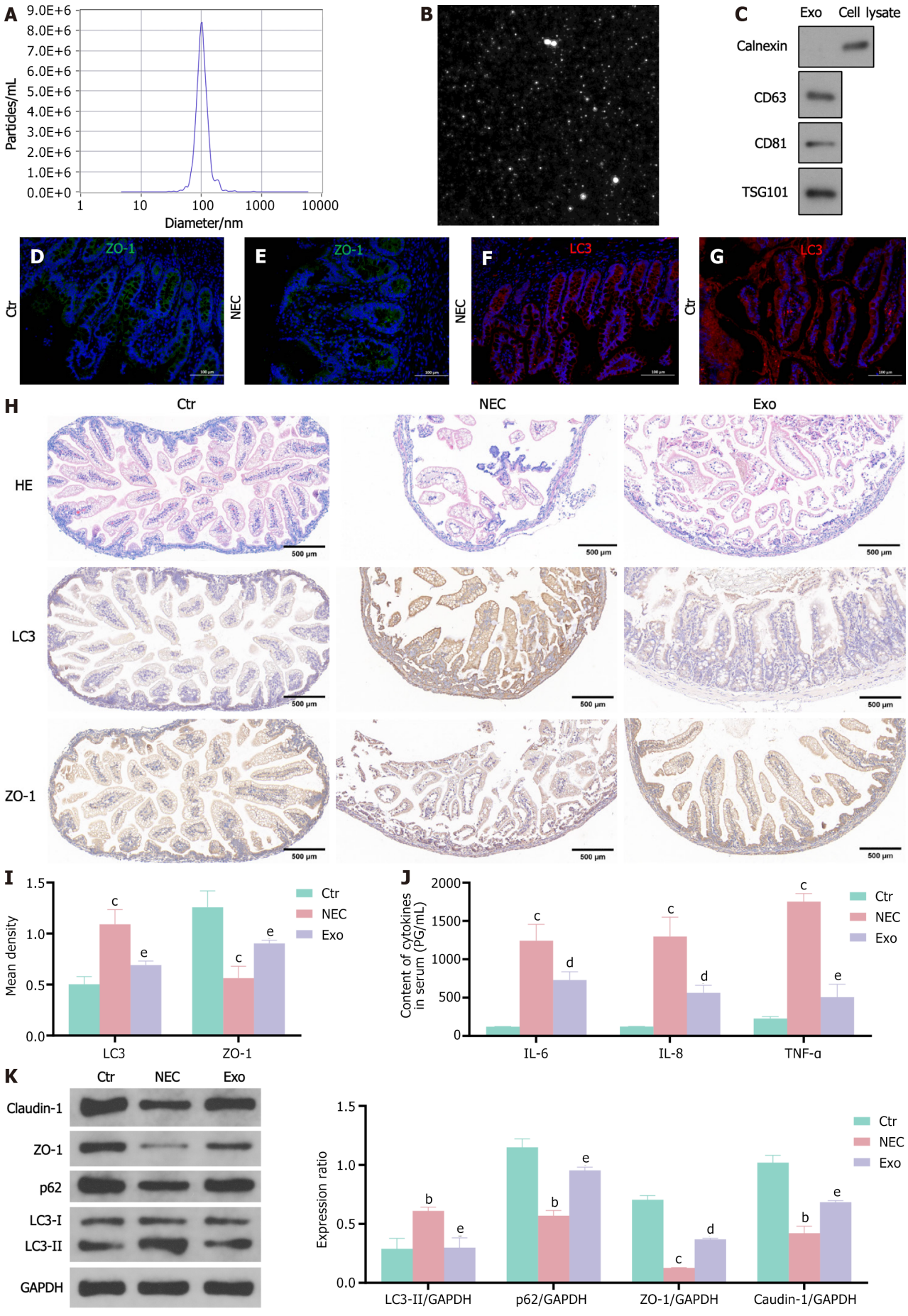
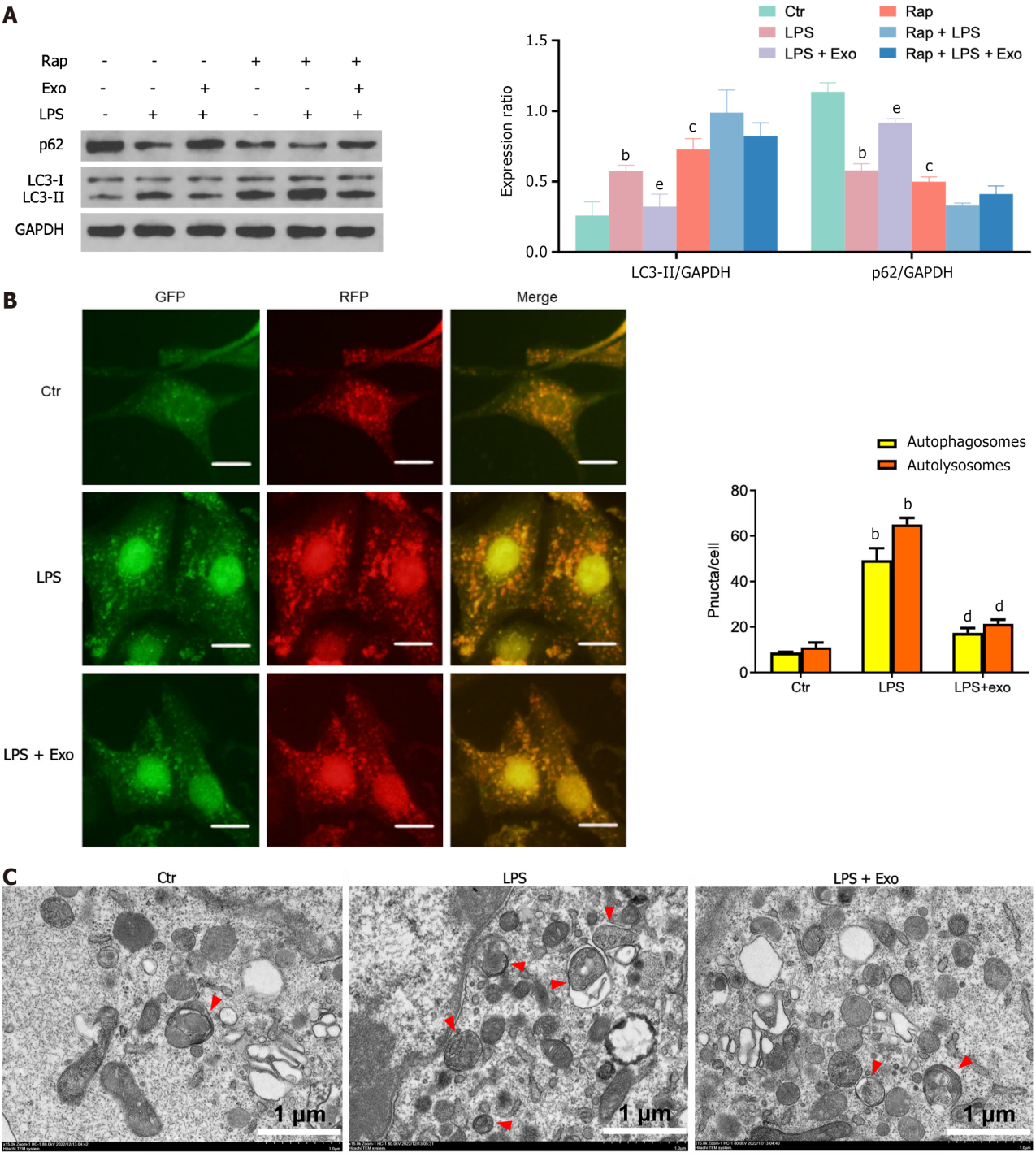


Figure 1 Intestinal epithelial cells autophagy increases and tight junction protein expression decreases in necrotizing enterocolitis

neonates. Human umbilical cord mesenchymal stem cell exosomes reduce autophagy and increase tight junction protein expression in the small intestine tissue of necrotizing enterocolitis mice. A-G: Exosomes particle size analysis (nanoparticle tracking analysis method) analyzes the size of human umbilical cord mesenchymal stem cell exosomes (hUCMSCs-exos) vesicles (A); morphology of isolated exosomes using transmission electron microscopy (B); expression levels of TSG101, CD81, CD63, and calnexin in hUCMSCs-exos were detected by western blot (C); expression of the intestinal epithelial cell-specific protein ZO-1 in neonatal colon tissue (green fluorescence) (D and E); expression of the autophagy protein LC3 in intestinal epithelial cells in neonatal colon tissue (red fluorescence), scale bar, 100 μm (F and G); H and I: Hematoxylin-eosin and immunohistochemical staining of LC3 and p62 in mouse distal ileum. Scale bar = 500 μm; J: Inflammatory factor levels in mice serum was detected by enzyme-linked immunosorbent assay; K: Western blot detects autophagy (LC3, p62) and tight junction (ZO-1, claudin-1) protein expression in mouse distal ileum tissue. Data was expressed as mean ± SEM of three or six independent experiments. $^{b}P < 0.01$ *vs* control group, $^{c}P < 0.001$ *vs* control group, $^{d}P < 0.05$ *vs* necrotizing enterocolitis, $^{e}P < 0.01$ *vs* necrotizing enterocolitis. NEC: Necrotizing enterocolitis; Ctr: Control; IL: Interleukin; TNF: Tumor necrosis factor.



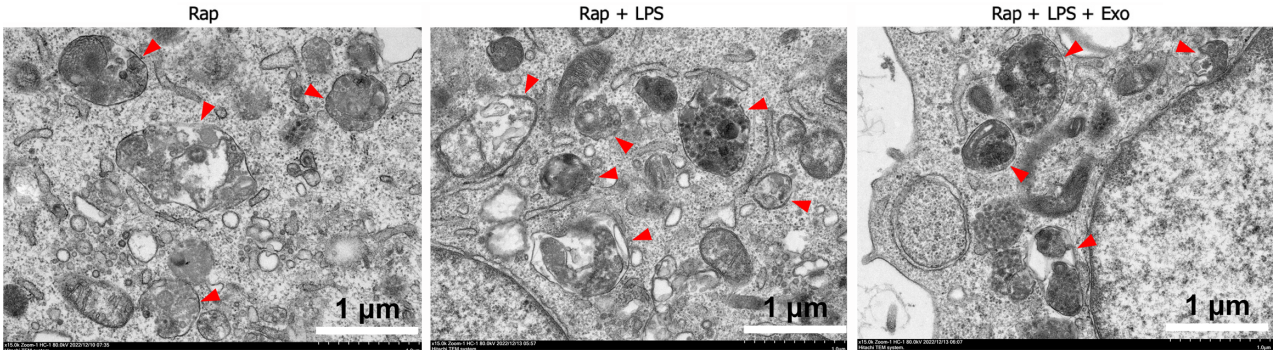


Figure 2 Human umbilical cord mesenchymal stem cell exosomes attenuate lipopolysaccharide-induced autophagy in intestinal epithelial cells. A: The expression of p62 and LC3 in each cell group was determined by western blotting; B: IEC-18 cells were infected with Ad-mRFP-GFP-LC3 for 48 h and photographed with the confocal microscopy. Representative mRFP-LC3, GFP-LC3 and merge images were shown. The number of GFP-LC3 dots and mRFP-LC3 dots per cell were counted to quantify the number of autophagosomes (yellow dots) and autolysosomes (red dots) per cell; C: The ultrastructure of IEC-18 cells was presented by transmission electron microscopy, with red arrows indicating degradative autophagic vacuoles. The scale bar represents 1 μm. Data was expressed as mean ± SEM of three or six independent experiments. $^{b}P < 0.01$ *vs* control group, $^{c}P < 0.001$ *vs* control group; $^{d}P < 0.05$ *vs* lipopolysaccharide, $^{e}P < 0.01$ *vs* lipopolysaccharide. LPS: Lipopolysaccharide.

employed in animal models of ischemia-reperfusion and NEC[8]. However, the specific mechanism of action is still unclear.

Exosomes, which are vesicles formed by the fusion of multivesicular bodies and plasma membranes, are secreted by various cells including stem cells. They serve as an efficient communication medium for transmitting genetic information between cells. Exosomes contain proteins and RNAs, which effectively regulate gene expression in target cells and impact their physiological state. In animal models, stem cells have been shown to primarily reduce the incidence of NEC through paracrine mechanisms. Stem cell-derived exosomes, which are bioactive factors secreted by stem cells, have the potential to achieve similar or even superior therapeutic effects[15]. Our study revealed that hUCMSC-exos significantly reduced plasma inflammatory factor levels, alleviated intestinal damage, and increased the expression of tight junction proteins in intestinal epithelial cells in NEC mice.

Autophagy plays a significant role in maintaining the balance of the gastrointestinal mucosal barrier. The regulation of intestinal epithelial connections helps maintain the homeostasis of the intestinal mucosal environment[4]. In NEC rats, typical autophagy phenomena have been observed in intestinal epithelial cells. Treatment with epidermal growth factor has been found to reduce autophagy and protect the intestines of NEC rats by decreasing the expression of Beclin-1 in ileal epithelial cells[16]. Similarly, vitamin D-3 has been shown to inhibit overactivated autophagy in intestinal epithelial cells of NEC rats, thereby promoting intestinal mucosal integrity[12]. Clinical studies have indicated that the autophagy protein Beclin-1 is highly expressed in neonatal NEC patients with severe intestinal necrosis, while the autophagy protein p62 is expressed at low levels. Furthermore, genetic mutations in the autophagy-related gene ATG16 have been found to be associated with NEC in premature infants, suggesting that reduced autophagy caused by these mutations may have a protective effect on NEC[13]. Similarly, we found that the expression of the autophagy marker protein LC3 was significantly increased in the colon tissue of neonates with NEC. Moreover, immunohistochemistry and western blot analyses revealed an increase in autophagy in the small intestinal tract of NEC mice, while the presence of hUCMSC-exos significantly suppressed autophagy in intestinal tissue. However, whether autophagy regulation by hUCMSC-exos is involved in the protective effect of hUCMSC-exos on NEC is unclear.

Specifically, excessive autophagy in intestinal epithelial cells can hinder cell migration, damage the intestinal barrier, and increase intestinal permeability by degrading tight junction proteins such as claudin-210. The autophagy-inducing factor rapamycin has been shown to significantly inhibit the migration of various cells, including intestinal epithelial cells, both *in vitro* and *in vivo*[17]. Therefore, we verified the effects of hUCMSC-exos on autophagy, migration and functional proteins in intestinal epithelial cells *in vitro*.

We found that hUCMSC-exos can reduce the expression of autophagy and inflammatory factors induced by LPS in IEC-18 cells. Additionally, hUCMSC-exos increased the expression of tight junction proteins and cell proliferation proteins and enhanced cell migration. However, when autophagy is activated by Rap, the aforementioned effects of hUCMSC-exos are weakened. These results highlight the role of autophagy in mediating the protective effect of hUCMSC-exos on intestinal epithelial cells. Considering the significance of intestinal epithelial cells in the development and progression of NEC, these findings strongly suggest that autophagy may play a crucial role in alleviating NEC caused by hUCMSC-exos.

CONCLUSION

In conclusion, we provide concrete evidence that downregulated autophagy in intestinal epithelial cells reduces intestinal inflammation and damage in NEC mice, and that targeting autophagy, such as *via* hUCMSC-exos, could serve as a potential therapeutic strategy for modulating NEC pathogenesis. The specific mechanism by which exosomes regulate

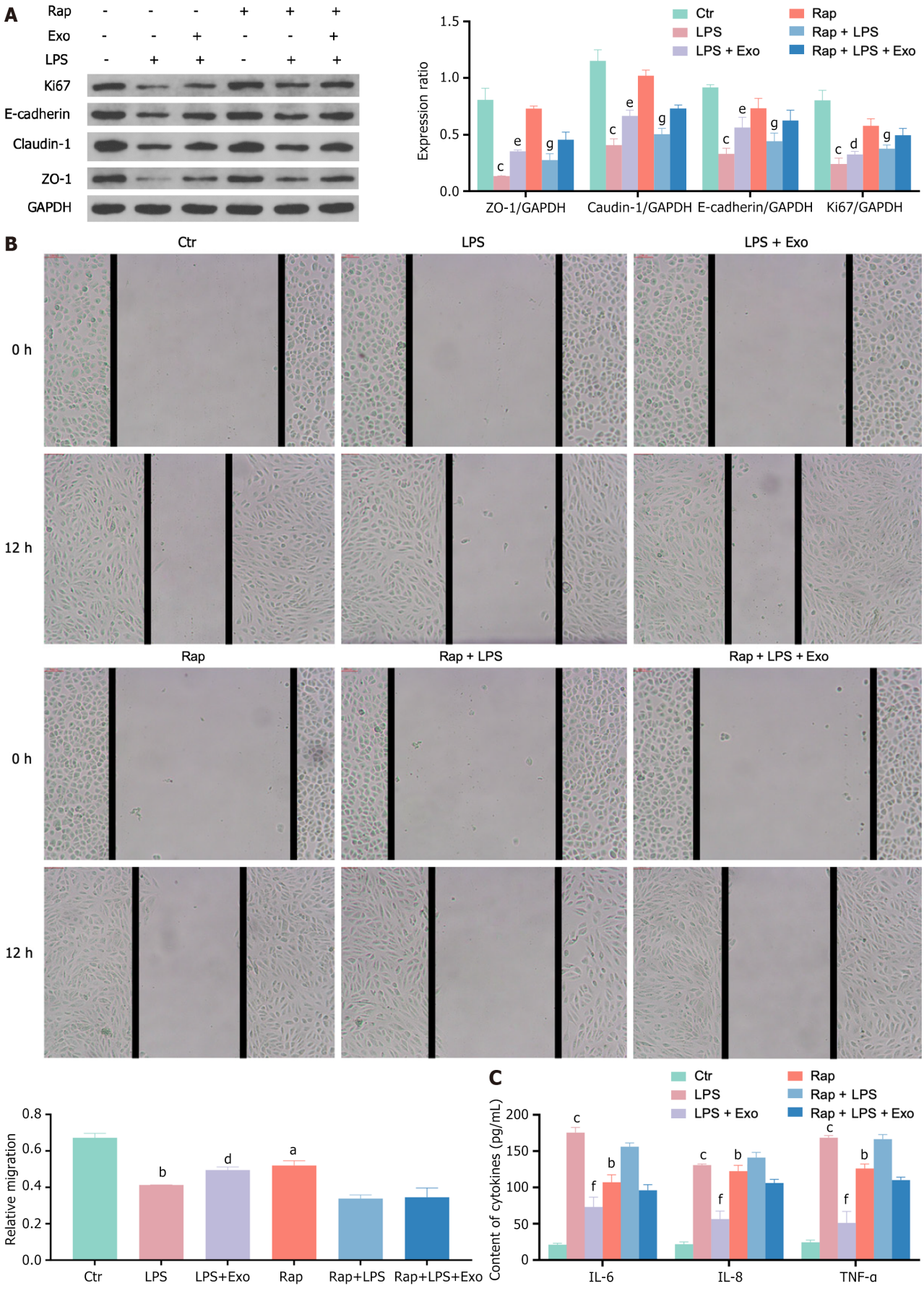


Figure 3 Human umbilical cord mesenchymal stem cell exosomes attenuate inflammation and injury induced by lipopolysaccharide and enhance the migration ability of intestinal epithelial cells by regulating autophagy. A: The expression of tight junction proteins (ZO-1, claudin-1 and E-cadherin) and cell proliferation proteins in each cell group was determined by western blotting; B: Migration ability of IEC-18 was detected by wound healing assay;

C: Inflammatory factor levels in cell supernatants were detected by enzyme-linked immunosorbent assay. Data was expressed as mean ± SEM of three or six independent experiments. [a]*P* < 0.05 *vs* control group, [b]*P* < 0.01 *vs* control group, [c]*P* < 0.001 *vs* control group; [d]*P* < 0.05 *vs* lipopolysaccharide, [e]*P* < 0.01 *vs* lipopolysaccharide, [f]*P* < 0.001 *vs* lipopolysaccharide; [g]*P* < 0.01 *vs* Rap. LPS: Lipopolysaccharide; Ctr: Control; IL: Interleukin; TNF: Tumor necrosis factor.

autophagy is still unclear, and the components of exosomes are complex. Our main research focus in the future will be to clarify which components play a major role in the aforementioned effects of exosomes.

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FOOTNOTES

Author contributions: Zhu L and Li N designed and coordinated the study; Zhu L, He L, and Duan W performed the experiments, acquired and analyzed data; Zhu L wrote the manuscript; Li N and Yang B contributed to the editing, polishing, and data processing of this manuscript. Li N and Yang B contributed equally as corresponding author, and all authors approved the final version of the article.

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REFERENCES

1 **Alsaied A**, Islam N, Thalib L. Global incidence of Necrotizing Enterocolitis: a systematic review and Meta-analysis. *BMC Pediatr* 2020; **20**: 344 [PMID: 32660457 DOI: 10.1186/s12887-020-02231-5]
2 **Frost BL**, Modi BP, Jaksic T, Caplan MS. New Medical and Surgical Insights Into Neonatal Necrotizing Enterocolitis: A Review. *JAMA Pediatr* 2017; **171**: 83-88 [PMID: 27893069 DOI: 10.1001/jamapediatrics.2016.2708]
3 **Wei J**, Meng Z, Li Z, Dang D, Wu H. New insights into intestinal macrophages in necrotizing enterocolitis: the multi-functional role and promising therapeutic application. *Front Immunol* 2023; **14**: 1261010 [PMID: 37841247 DOI: 10.3389/fimmu.2023.1261010]
4 **Yamoto M**, Lee C, Chusilp S, Yazaki Y, Alganabi M, Li B, Pierro A. The role of autophagy in intestinal epithelial injury. *Pediatr Surg Int* 2019; **35**: 1389-1394 [PMID: 31555857 DOI: 10.1007/s00383-019-04566-2]
5 **Bel S**, Hooper LV. Secretory autophagy of lysozyme in Paneth cells. *Autophagy* 2018; **14**: 719-721 [PMID: 29388875 DOI: 10.1080/15548627.2018.1430462]
6 **Guan Y**, Zhang L, Li X, Zhang X, Liu S, Gao N, Li L, Gao G, Wei G, Chen Z, Zheng Y, Ma X, Siwko S, Chen JL, Liu M, Li D. Repression of

Mammalian Target of Rapamycin Complex 1 Inhibits Intestinal Regeneration in Acute Inflammatory Bowel Disease Models. *J Immunol* 2015; **195**: 339-346 [PMID: 26026060 DOI: 10.4049/jimmunol.1303356]

7 **Markel TA**, Martin CA, Chaaban H, Canvasser J, Tanner H, Denchik H, Good M. New directions in necrotizing enterocolitis with early-stage investigators. *Pediatr Res* 2020; **88**: 35-40 [PMID: 32855511 DOI: 10.1038/s41390-020-1078-0]

8 **Drucker NA**, Te Winkel JP, Shelley WC, Olson KR, Markel TA. Inhibiting hydrogen sulfide production in umbilical stem cells reduces their protective effects during experimental necrotizing enterocolitis. *J Pediatr Surg* 2019; **54**: 1168-1173 [PMID: 30879750 DOI: 10.1016/j.jpedsurg.2019.02.037]

9 **Yang J**, Chen Z, Pan D, Li H, Shen J. Umbilical Cord-Derived Mesenchymal Stem Cell-Derived Exosomes Combined Pluronic F127 Hydrogel Promote Chronic Diabetic Wound Healing and Complete Skin Regeneration. *Int J Nanomedicine* 2020; **15**: 5911-5926 [PMID: 32848396 DOI: 10.2147/IJN.S249129]

10 **Ma F**, Hao H, Gao X, Cai Y, Zhou J, Liang P, Lv J, He Q, Shi C, Hu D, Chen B, Zhu L, Xiao X, Li S. Melatonin ameliorates necrotizing enterocolitis by preventing Th17/Treg imbalance through activation of the AMPK/SIRT1 pathway. *Theranostics* 2020; **10**: 7730-7746 [PMID: 32685016 DOI: 10.7150/thno.45862]

11 **Zhu L**, Duan W, Wu G, Zhang D, Wang L, Chen D, Chen Z, Yang B. Protective effect of hydrogen sulfide on endothelial cells through Sirt1-FoxO1-mediated autophagy. *Ann Transl Med* 2020; **8**: 1586 [PMID: 33437785 DOI: 10.21037/atm-20-3647]

12 **Chen L**, Lv Z, Gao Z, Ge G, Wang X, Zhou J, Sheng Q. Human β-defensin-3 reduces excessive autophagy in intestinal epithelial cells and in experimental necrotizing enterocolitis. *Sci Rep* 2019; **9**: 19890 [PMID: 31882811 DOI: 10.1038/s41598-019-56535-3]

13 **Sampath V**, Bhandari V, Berger J, Merchant D, Zhang L, Ladd M, Menden H, Garland J, Ambalavanan N, Mulrooney N, Quasney M, Dagle J, Lavoie PM, Simpson P, Dahmer M. A functional ATG16L1 (T300A) variant is associated with necrotizing enterocolitis in premature infants. *Pediatr Res* 2017; **81**: 582-588 [PMID: 27893720 DOI: 10.1038/pr.2016.260]

14 **Di SJ**, Wu SY, Liu TJ, Shi YY. Stem cell therapy as a promising strategy in necrotizing enterocolitis. *Mol Med* 2022; **28**: 107 [PMID: 36068527 DOI: 10.1186/s10020-022-00536-y]

15 **McCulloh CJ**, Olson JK, Wang Y, Zhou Y, Tengberg NH, Deshpande S, Besner GE. Treatment of experimental necrotizing enterocolitis with stem cell-derived exosomes. *J Pediatr Surg* 2018; **53**: 1215-1220 [PMID: 29661576 DOI: 10.1016/j.jpedsurg.2018.02.086]

16 **Maynard AA**, Dvorak K, Khailova L, Dobrenen H, Arganbright KM, Halpern MD, Kurundkar AR, Maheshwari A, Dvorak B. Epidermal growth factor reduces autophagy in intestinal epithelium and in the rat model of necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol* 2010; **299**: G614-G622 [PMID: 20539009 DOI: 10.1152/ajpgi.00076.2010]

17 **Neal MD**, Sodhi CP, Dyer M, Craig BT, Good M, Jia H, Yazji I, Afrazi A, Richardson WM, Beer-Stolz D, Ma C, Prindle T, Grant Z, Branca MF, Ozolek J, Hackam DJ. A critical role for TLR4 induction of autophagy in the regulation of enterocyte migration and the pathogenesis of necrotizing enterocolitis. *J Immunol* 2013; **190**: 3541-3551 [PMID: 23455503 DOI: 10.4049/jimmunol.1202264]

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