Intestinal Epithelial Cell Derived Exosomes Package MicroRNA-23a-3p Alleviate Gut Damage After Ischemia/Reperfusion via Targeting MAP4K4

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Research

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Abstract

Backgroud:

Intestinal epithelial cells (IEC) contribute to regulate gut injury after intestinal isochemia/reperfusion (II/R). Exosomes are well documented to deliver bioactive molecules to recipient cells to modulate cell function. However, the role of IEC-derived exosomes in gut damage after II/R and the underlying mechanisms remains unclear.

Methods

IEC-derived exosomes were intravenously injected into the rats after the superior mesenteric artery occlusion for 45 min following reperfusion for 6h. The scores of gut injury, ZO-1 and IL-17A expression, TNF- α and endotoxin concentration were determined at 3 days after II/R assault. Exosomal microRNA-23a-3p and its downstream target molecule-MAP4K4 were also detected.

Results

Our results showed that the IEC-derived exosomes attenuated the damage of IECs after oxygen-glucose deprivation *in vitro* ($p < 0.05$) and the degree of gut injury at 3 days after II/R assault *in vivo* ($p < 0.05$). Injection of miR-23a-3p knockdown exosomes derived from IECs aggravated the II/R injury whereas PF-6260933, a small-molecule inhibitor of MAP4K4, was partly reversed the injured degree by intraperitoneal injection. Underlying mechanism studies revealed that exosomal miR-23a-3p attenuated gut damage by regulating its downstream target MAP4K4.

Conclusions

We demonstrated that IEC-derived exosomes alleviated the II/R injury and promoted gut damage recovery via exosomal miR-23a-3p and its downstream target molecule-MAP4K4.

Introduction

Intestinal ischemia/reperfusion (II/R)-induced injury is one of the most serious clinical events which result from different pathophysiologic conditions, including burns, trauma, haemorrhagic shock, infection, acute superior mesenteric artery (SMA) occlusion, as well as organ transplantation, which leads to severe injuries in the structure and function of intestinal epithelial cells (IECs) [1–2]. It is an intense local inflammation responses that is produced in the small intestine transiently deprived of blood flow during reperfusion[3–5]. More complex mechanisms that trigger inflammation are involved in its development and amplification have been identified only partially [6–8]. Extracellular vesicles have been participated in regulating the inflammatory injury of IECs, including the local immuno-inflammatory responses, cell

apoptosis, structure of tight junction (TJ) and TJ proteins[9–10]. However, it remains unclear that the underlying mechanism of extracellular vesicles regulating the function of IECs.

Exosomes are membranous vesicles that are secreted by different type of cells including IECs affected by II/R injury [11]. Evidences show that exosomes have been implicated in mediating the functions of cells in the micro-environment, including immune and inflammatory reactions, cell-to-cell crosstalk and substance transfer between cells, cell trafficking, proliferation and differentiation [12–13]. Additionally, Exosomes contains all kinds of mRNA, microRNA (miRNA), DNA and proteins, which contribute to the transfer between cells and affect the function of the target cells. Among them, miRNA has been shown to participate in various biological and pathological processes [14].

Recent studies have shown that exosomes have been represented as a protective role in I/R injury by transmitting cargo to the recipient cells. M2 microglia-derived exosomes enriched with the miRNA-124 have been reported to attenuate the I/R brain injury and promoted neuronal survival via the transfer of the exosomes into neurons [15]. The exosomes secreted by the bone marrow mesenchymal stem cells or the plasma have been reported to reduced infarct size and provide protection against myocardial I/R injury by delivering endogenous protective signals to the myocardium [16–17]. A recent study showed that exosomal miR-124-3p derived from bone marrow mesenchymal stem cells attenuated nerve injury induced by spinal cord I/R injury by regulating Ern1 and M2 macrophage polarization [18].

However, not much is known about the effect of intestinal epithelial cell derived exosomes (IEC-exo) on the function of small intestinal epithelial cells after II/R. In the present study, we aimed to verify and elucidate the roles of IEC-exo in the gut injury after II/R and its possible mechanism which is related to exosomal miRNA-23a-3p and its corresponding downstream genes.

Materials And Methods

II/R Model and Treatment

Male SD rats were purchased from SBF Biotechnology Co., Ltd (Beijing, China) and maintained under specific pathogen-free (SPF) conditions. The rats were supplied a standard chow until they reached the desired body weight (180-200g). The anesthetic management and II/R model establishment were performed as following: Briefly, the rats were fasted, but allowed free access to water, 12 h before the experiment. After induction of anaesthesia with pentobarbital sodium (50 mg/kg body weight) by intraperitoneal injection. After midline laparotomy, the superior mesenteric artery (SMA) was isolated and blocked with a microvascular clamp until complete ischemia of SMA for 45min. The clamp was then removed to allow for reperfusion for 6h and further the exosomes or miRNA was injected into the tail vein of II/R rats for 3 consecutive days. Three days after II/R, the rats were sacrificed and the small intestines were taken out. The rats in the II/R group were treated with PBS. In sham-operation group, the SMA was isolated without clamping and was exposed to the same procedure as rats with SMA occlusion. All procedures were performed according to the national guidelines for the treatment of animals and were approved by the Institutional Ethics Committee of Nanjing Medical University (Nanjing, China).

Study Design

A total of 42 male SD rats were randomly divided into the following 7 groups, 6 rats in each group: 1) Sham-operation group (Sham): rats that underwent sham surgery; 2) II/R group (II/R): Phosphate buffered saline (PBS) at the dosage of 2ml/200g was injected into the tail vein of the II/R rats for 3 consecutive days; 3) II/R plus IEC-exo treated group (II/R+exo): exosomes derived from IEC-18 were injected through the tail vein of the II/R rats for 3 consecutive days; 4) II/R plus IEC-exo combined with miR-23a-3p treated group (II/R+exo+miR): exosomes derived from IEC-18 combined with miR-23a-3p was injected into the tail vein of the II/R rats for 3 consecutive days; 5) miRNA vector treated group (II/R+miR-cn-exo): IEC-18 were treated with a control lentivirus vector (no-load shRNA lentivirus), and exosomes were isolated and injected through the tail vein for 3 consecutive days; 6) II/R plus miR-23a-3p knockdown IEC-exo treated group (II/R+miR-k/d-exo): miR-23a-3p knockdown lentivirus was transfected into IEC-18, and then exosomes were isolated and injected through the tail vein for 3 consecutive days; 7) II/R plus miR-23a-3p k/d IEC-exo combined with PF-6260933 (Selleck biological Co., Ltd, USA) treated group (II/R+miR-k/d exo+PF-6260933): Exosomes were isolated from IEC-18 transfected by miR-23a-3p knockdown lentivirus and injected through the tail vein and meanwhile, given an intraperitoneal injection of $20\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ PF-6260933 for 3 consecutive days. Treatment protocol: about 6.67×10^5 of exosomes at the dosage of 2ml/200g in each rat per day were injected after intestinal ischemia for 45min following reperfusion for 6h for 3 consecutive days. $5\mu\text{g/g}$ of miR-23a-3p at the dosage of 2ml/200g in each rat was administered for 3 consecutive days after II/R. Three days later, rats were sacrificed and blood samples were collected by cardiac puncture. Rats from each group were used for staining or protein and total RNA extraction.

IEC-18 Culture

Primary cells from the rat intestinal epithelial cell line No. 18 (IEC-18) were purchased from the Beina biological Co., Ltd (Beijing, China). The cryopreservation tube containing 1 ml cell suspension was rapidly shaken and thawed in 37°C water bath, and 5 ml medium was added to mix evenly. Centrifuge for 5 min at 1000 rpm. the supernatant was discarded and added 4-6 ml of the complete medium (Dulbecco's modified Eagle's medium including 0.1Unit/ml bovine insulin and 10% FBS). Then all cell suspensions were added to the culture bottle for overnight culture and cultured in a humidified incubator at 37°C in a humidified atmosphere with 5% CO_2 overnight. If the cell density reaches 80% - 90%, it can be used for further experiments. All experiments used cells at the 4th to 6th passage and were performed in at least triplicate.

Oxygen-Glucose Deprivation (OGD) and Reoxygenation Model

According to the literature[19], primary IEC-18 cells were collected, digested and centrifuged, and then planted in Petri dishes (Corning, Tewksbury, USA). They were divided into the control group (Con), the OGD group (OGD), IEC derived-exosome treated group (OGD+exo), IEC derived-exosome plus miR-23a-3p treated group (OGD+exo+miR), miRNA vector treated group (OGD+miR-cn-exo) and miR-23a-3p

knockdown IEC-exo treated group (OGD + miR-k/d-exo), 3 repetitions per group. After the cells were cultured for 12h and adhered to the wall, and exchanged by cobalt chloride (GoCl₂) solution with a final concentration of 10μmol/L in the OGD group. After the cells were treated for 4h, the culture medium was discarded, cleaned with PBS for 3 times, and the culture medium without GoCl₂ solution was replaced again. After 72 h of completing the reoxygenation induction protocol, cells and medium were collected for cell viability, cytokines and exosome microRNA assays. 0.03μmol/L of miR-23a-3p with or without 2 × 10⁶ of exosomes derived from IECs or miR-23a-3p knockdown IECs were added into the Petri dishes once at 4 h after OGD.

Cell viability assay

Cell viability was quantitatively analyzed by a 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) assay (KGA311-KGA312, Keygen Biotech Co. China). Briefly, the experiments were conducted in 96-well plates, and then MTT and Methyl sulfoxide (DMSO) were added into the plates in turn. Finally, the absorbance of each individual well was determined at 490 nm.

Lactate dehydrogenase (LDH) Assay

IEC-18 cellular injury was quantified by the amount of LDH released. The determination of LDH concentrations in the culture medium of primary IEC-18 cells was measured using the enzyme-linked immunosorbent assay (ELISA) kit (Senbenjia Biotech Co. Nanjing, China) according to the manufacturer's protocol. Briefly, both purified rat LDH and LDH were added into to the coat microtiter plate wells, and then labeled with HRP. After washing completely, TMB substrate solution was added and TMB substrate became blue color at HRP enzyme-catalyzed. Reaction was terminated by the addition of a sulphuric acid solution and the color change was measured at 450 nm using a microplate reader. The concentration of LDH is determined by comparing the OD values to the standard curve.

Exosome Isolation and Identification

Exosomes were isolated from the conditioned IEC-18 culture medium. Prior to culture medium collection, IEC cells were washed twice with PBS, and the medium was switched to exosome-free medium. The cells were then cultured for 48 h. The supernatant was collected and went through sequential centrifuged at 300g for 5min, 1200g for 20min and 10000g for 30min at 4 °C to discard the sediment. The supernatant was collected and centrifuged at 110000g for 60min at 4 °C and the precipitate was the exosomes. The exosomes were collected and fixed with the glutaraldehyde electron microscope fixing solution (composed of glutaraldehyde, phosphate and deionized water, pH 7.2 ~ 7.4), and then sent to a transmission electron microscope (Thermo Scientific, USA) for detection. Nanoparticle tracking analysis (NTA, Brookhaven, New York) was used to measure exosome diameter and particle number. The protein content was measured using BCA protein assay, and exosome markers ALIX, CD63, HSP70 and tumor susceptibility gene 101 (TSG101) were detected by western blot analysis.

Western Blot

Western blot analysis of exosome markers and MAP4K4 was performed. Samples from IEC-18 cells and small intestinal tissues were collected from the ipsilateral hemisphere and quantified with the BCA protein assay (Biyuntian, China). The protein lysate was mixed with 5× loading buffer (Biyuntian, China) and boiled at 95°C for 5 min, then loaded into the SDS-PAGE gel (Biyuntian, China). After electrophoresis, the separated proteins were transferred to PVDF membranes (Merck Millipore, German). The membrane was blocked with 5% skim milk at room temperature for 1 h and then incubated with the following primary antibodies at 4°C overnight: anti-HSP70 (1:1000, ab181606, Abcam, Cambridge, MA), anti-ALIX(1:1000, ab186429, Abcam), anti-CD63 (1:1000, ab217345, Abcam), anti-TSG101(1:1000, ab125011, Abcam), anti-MAP4K4 (1:100, ab80418, Abcam), GAPDH and β -actin (BM0627, Boshide, China). The membrane was then washed three-times and incubated with secondary antibody at room temperature for 1 h. ECL reagent (Biyuntian, China) were used for chemiluminescent detection.

Lentiviral Vector Transduction

3×10^5 cells per well of IECs in 6-well plates were transduced with 5×10^8 TU/ml of miR-23a-3p knockdown lentivirus (Shanghai Genechem Co., Ltd, China) in Dulbecco's modified Eagle's medium (HyClone), and incubated at 37°C in 5% CO₂. Three days after transduction green fluorescent protein signal was observed by fluorescence microscopy. No-load shRNA lentivirus was used as a control.

Hematoxylin-Eosin (HE) Staining

After the rats were sacrificed, specimens from the small intestines (5cm from the distal end of ileum) were taken out and fixed with 10% formaldehyde. The paraffin sections were stained with hematoxylin and eosin for histological evaluation in a single blinded fashion. The degree of gut injury was evaluated according to the Park Chiu score [20]: Grade 0: Normal mucosa; Grade 1: Subepithelial bleb at the tip of the villus; Grade 2: More extended subepithelial space (upper half of the villus); Grade 3: Epithelial lifting down to the base of the villus, occasional epithelial breakdown; Grade 4: Frequent denuded villi; Grade 5: Loss of villous tissue; Grade 6: Crypt layer destruction; Grade 7: Transmucosal infarction; Grade 8: Transmural infarction. For evaluation of lesions, 10 arbitrary microscopic fields were viewed in each sample.

Immunohistochemistry

Immunohistochemistry was performed according to the manufacturer's instructions. Briefly, the small intestinal tissues were taken from the rats and immersed in 4% paraformaldehyde. Intestinal sections (4 μ m) at 1.5cm from ileocecal part were microtomed. Slides were then blocked with 5% donkey serum for 1 h at room temperature before being incubated with the primary Zona Occludens-1 (ZO-1) antibodies (1:200, Boshide Co., China). Sections were then incubated with appropriate secondary antibodies for 4 h at room temperature. Diaminobenzidine 3,3'-diaminobenzidine was used to stain the sections. After neutral resin sealing, sections were imaged using an optical microscope.

Quantitative Real-Time PCR

Total RNA from the IEC-18 cells derived exosomes and the small intestinal tissues of rats was extracted by Trizol reagent (Invitrogen, Carlsbad, CA) and isolated according to the manufacturer's manual. Single-strand cDNA was synthesized using a universal cDNA synthesis kit (Qiagen, Hilden, Germany). Primer sequences of miRNA or RNA were as follows: miRNA-23a-3p, Forward: AUCACAUUGCCAGGGAUUUCC, Reverse: AUCACAUUGCCAGGGAUUU CC. IL-17A, Forward: CCGTTCCACTTCACCCT, Reverse: GGTCCAACCTT CCCCTCA. ZO-1, Forward: TGAGCCTTGAACCTTGACC, Reverse: GGGCA CAGCATTGTATCA. The expression of miRNA was performed using a real-time PCR system with the following cycling conditions: 95°C for 10 min followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. The comparative cycle threshold method ($2^{-\Delta\Delta CT}$) was applied for the relative expression of each miRNA expression and GPADH is as the control.

Tumor necrosis factor- α (TNF- α) and endotoxin level detection

Rat TNF- α activity and endotoxin concentration in the media were assayed using ELISA kit (Saimike Biotech Co. Chongqing, China). Both purified rat TNF- α or endotoxin antibody and TNF- α or endotoxin were added to the coat microtiter plate wells and then labeled with HRP. After washing completely, TMB substrate solution was added and TMB substrate becomes blue color at HRP-enzyme-catalyzed. Finally, the reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450nm. The concentration of endotoxin was determined by comparing the OD values to the standard curve.

Luciferase Reporter Assay

In order to verify the relationship between miR-23a-3p and MAP4K4, we inserted the 3'UTR region of MAP4K4 into a vector and transfected the vector into 293T cells. Additionally, we performed site-directed mutagenesis to further determine the site of action of the miRNA and the 3' UTR of MAP4K4. Luciferase activity was determined with a Dual-Luciferase Reporter Assay Kit using a Dual-Light Chemiluminescent Reporter Gene Assay System (Promega, Fitchburg, WI) and was normalized to the Renilla (ObiO Co., Ltd, Shanghai, China) luciferase. The rat MAP4K4 3'UTR was amplified and cloned into a pGL4 vector containing the firefly luciferase reporter gene (ObiO Co., Ltd, Shanghai, China). For the luciferase assay, 293T cells were cotransfected with 100ng firefly luciferase constructs, 10 ng pRL-TK Renilla luciferase plasmid, and 100 nmol/L synthetic miR-23a-3p mimic and no-load vector. The results are expressed as relative luciferase activity (Firefly luciferase/Renilla luciferase).

Immunofluorescence staining

Samples were separated from the small intestinal tissues of rats, which further were frozen and cut into 5- μ m sections for immunofluorescent staining. After incubation with rabbit against rat MAP4K4 antibodies (Abcam Co. UK) at 4°C overnight. Sections were then incubated with appropriate secondary antibodies for 4 h at room temperature. The sections were washed with PBS, and 4', 6-diamidino-2-phenylindole (DAPI, Beyotime, Shanghai, China) was added and incubated for 10 minutes at room temperature. After washing with PBS, sections was imaged using a fluorescence microscope.

Statistical Analysis

Data were presented as the mean $\pm$ SD and analyzed using SPSS 21.0 (GraphPad Software, La Jolla, CA). Statistical analysis was performed by one-way analysis of variance (ANOVA). Difference was considered statistically significant when $P < 0.05$.

Results

Exosomes isolation and miRNA detection

Exosomes were then isolated from IEC-18 cells by ultracentrifugation and identified using a transmission electron microscope (TEM), nanoparticle tracking analysis (NTA) and western blot analysis. As shown in Figure 1C, the typical cup-shaped membrane vesicle morphology was observed, and the diameter of particles was in the range of 10-450.6 nm. Western blot results showed that exosome markers including ALIX, CD63, HSP70 and TSG101 were expressed in exosomes (Figure 1E). Further, exosomes were isolated and miR-23a-3p was detected after IECs were underwent OGD for 4 h and reoxygenation for 72 h. As shown in Figure 1F, exosomal miR-23a-3p obviously enhanced after IECs subjected to OGD, suggesting that miR-23a-3p from IEC-derived exosomes may be involved in OGD injury.

IEC-exo decreased damage of IECs underwent OGD

To investigate whether IEC-18-derived exosomes are involved in the protection of IECs or not, exosomes derived from IEC-18 were incubated with the IEC-18 with or without OGD for 4 h and reoxygenation for 72 h. To quantify the effect of IEC-derived exosomes on IECs that underwent OGD, MTT assay was performed for rapid quantification of cell viability. Cellular damage was quantified by the amount of LDH released. As shown in Figure 2B and 2C, cellular injury after OGD treatment was significantly increased while cell survival was sharply decreased compared with the control group. When the addition of IEC-18 derived exosomes, cell viability of IEC-18 underwent OGD was in turn obviously increased and cellular damage was significantly reduced detected by the LDH assay, which suggesting exosomes derived from IECs protect against IECs subjected to OGD injury.

IEC-exo attenuated gut injury subjected to II/R

In order to observe the effects of IEC-derived exosomes on II/R injury *in vivo*, exosomes derived from IEC-18 were infused into the rats with II/R injury for 3 consecutive days. As shown in Figure 2E and 2F, The villi structure of small intestines was complete and the villi end and the lamina propria were not damaged in the sham group. After II/R assault, intestinal mucosal epithelial cells were damaged and shed. The lamina propria was exposed and the blood vessels of the lamina propria were dilated and congested. The damage of intestinal mucosal epithelial cells in the II/R plus exosome group was significantly less than that of the II/R group, however, the exposed lamina propria was still visible. In addition, expression of ZO-1 protein and mRNA was significantly higher in the groups treated with IEC-exo than that in the II/R group (Figure 2G and 2H) while IL-17 mRNA expression was obviously lower compared with II/R group (Figure

2L). Meanwhile, TNF- α levels and Endotoxin concentration in the peripheral blood in the II/R plus exosome group were also higher than those in the II/R group (Figure 2J and 2K).

Exosomal miR-23a-3p reduced damage of IECs

IEC-derived exosomes are enriched with miR-23a-3p, which is significantly upregulated in IECs underwent OGD for 4 h and reoxygenation for 72 h as shown in Figure 1F. Exosomes have been reported that they are able to deliver miRNA and further regulate the function of recipient cells. To demonstrate the protective effects of exosomal miR-23a-3p on IECs, we added both IEC-derived exosomes and miR-23a-3p to the medium of IECs underwent OGD. The data showed that cellular damage was obviously decreased detected by LDH release and cell viability was significantly increased compared with IECs treated by IEC-derived exosomes alone (Figure 3A and 3B). Further, we verified the cellular protection of miR-23a-3p by knocking down miR-23a-3p in IEC-exo. As shown in Figure 3D, transfection of lentivirus carrying miR-23a-3p-shRNA downregulated the expression of miR-23a-3p in IEC-exo. Then, we added miR-23a-3p-knockdown exosomes to the medium of IECs after OGD. The results showed that cellular injury was significantly increased and cell survival was obviously decreased compared with IECs treated by IEC-derived exosomes alone (Figure 3A and 3B), which suggesting that miR-23a-3p derived from IECs protect against IECs subjected to OGD injury *in vitro*.

MiR-23a-3p derived from IEC-exo decreased gut damage after II/R

To investigate the protective roles of exosomal miR-23a-3p in gut injury after II/R assault, both IEC-derived exosomes and miR-23a-3p were administered from tail vein in a rat model of the II/R injury. It has been showed in Figure 4A that miR-23a-3p expression in the small intestinal tissues was sharply reduced compared with II/R rats treated with IEC-derived exosomes alone. Although the submucosal blood vessels also had dilation and congestion, the intestinal mucosal epithelial injury and Park-Chiu scores were significantly reduced compared with II/R rats treated with IEC-derived exosomes alone (Figure 4E and 4F). Additionally, expression of ZO-1 protein and mRNA was significantly higher than that in the groups treated with IEC-exo alone (Figure 4H and 4I). Meanwhile, TNF- α levels and Endotoxin concentration in the peripheral blood were also higher than those in the groups treated with IEC-derived exosomes alone (Figure 4J and 4K). Further, miR-23a-3p-knock-down exosomes derived from IECs were infused to the II/R rats from tail vein, miR-23a-3p expression in the gut was significantly lower than that in the groups treated with IEC-derived exosomes alone (Figure 4A). It had been showed in Figure 4E that intestinal mucosa necrosis and shedding, part of the lamina propria is exposed, mucosa and submucosa hyperemia and hemorrhage and there existed shedding necrosis mucosal epithelial cells and inflammatory cells in the intestinal cavity. Park-Chiu scores were significantly higher than that in the groups treated with IEC-derived exosomes alone (Figure 4F). Expression of ZO-1 protein and mRNA was significantly lower than that in the groups treated with IEC-exo alone (Figure 4H and 4I) while IL-17A levels were obviously higher than that in the groups treated with IEC-exo alone (Figure 4L). Meanwhile, TNF- α levels and Endotoxin concentration in the peripheral blood were also higher than those in the groups

treated with IEC-derived exosomes alone (Figure 4J and 4K), which suggesting that miR-23a-3p derived from IECs protect against the gut subjected to II/R injury *in vivo*.

Exosomal miR-23a-3p attenuated gut damage by regulating its downstream target MAP4K4

To demonstrate that IEC-derived exosomes alleviated gut damage regulated by exosomal miR-23a-3p and its downstream target molecule, Sterile-20-like mitogen-activated protein kinase kinase kinase 4 (MAP4K4) as a potential downstream target of miR-23a-3p was selected from TargetScan and miRBase (Figure 5A). The dual luciferase assay indicated that MAP4K4 is the direct downstream target of miR-23a-3p which contains two binding sites (Figure 5A and 5C). Immunofluorescence staining and western blot analysis demonstrated that MAP4K4 expression in the small intestinal tissues was significantly upregulated in the groups treated with exosomes derived from miR-23a-3p knockdown lentivirus transfected IECs compared with the groups treated with no-load shRNA lentivirus (Figure 5D and 5E). Expression of MAP4K4 was substantially decreased in the groups treated with miR-23a-3p knockdown exosomes plus PF-6260933, a small-molecule inhibitor of MAP4K4 (Figure 5D and 5E), because PF-6260933 inhibited upregulation of MAP4K4 expression caused by miR-23a-3p downregulation in the II/R rats treated with miR-23a-3p knockdown exosomes. The gut damage, Park-Chiu scores and IL-17A expression were significantly lower than that in the groups treated with miR-23a-3p knockdown exosomes alone (Figure 4E, 4F and 4L) accompanied by the decreased levels of TNF- α and endotoxin in the blood (Figure 4J and 4K), which suggesting that exosomal miR-23a-3p improved gut damage by regulating its downstream molecule-MAP4K4.

Discussion

Studies have revealed that extracellular vesicles secreted from intestinal epithelial cells are critical for mediating inflammation and repair as well as immune responses by transporting bioactive effectors and genetic information to the recipient cells [21–22]. Hsu et al reported that the IEC-6 cells affected by I/R injury can secrete exosomes and release paracrine mediators such as miRNAs, which induce cortical neuron apoptosis, necroptosis and pyroptosis [11]. Exosomes derived from IECs affected by carnosine were found to be involved in the activation of neuronal cells as mediators of brain-gut interaction via exosomal miR-6769-5p and its target gene ATXN1 [23]. In addition, exosomes released from IECs upon *C. parvum* infection can activate immune cells by shuttling various effector molecules, which may be relevant to host systemic responses to *Cryptosporidium* infection [24]. It is speculated that the IEC-derived exosomes may play an important role in regulating inflammation and immune reaction in the gut damage. In this study, LDH assay was performed so that we can detect the effects of IEC-derived exosomes on IECs that underwent 72h of reoxygenation following 4h of OGD. We found that IEC-derived exosomes decreased the levels of LDH releases compared with OGD treatment, which suggesting that IEC-derived exosomes significantly decreased IECs damage after OGD *in vitro* (Figure 2B). Additionally, in order to investigate whether the IEC-derived exosomes after 3 consecutive days of tail vein injection affects the intestinal epithelial function during the II/R development, the scores of gut damage were counted and the tight junction protein-ZO-1 and inflammatory factor-IL-17A were also examined. We

examined 5 slices of small intestinal tissues from each of 6 rats in each group and counted the scores and the protein or mRNA expression of ZO-1 and IL-17A in 5 regions from each slice of small intestinal tissues. The results showed that the damage scores were significantly lower in the groups treated with IEC-exo than that in the II/R groups (Figure 2F) and the expression of ZO-1 mRNA and protein was obviously higher in the groups treated with IEC-exo than that in the II/R groups (Figure 2H and 2I), which suggesting that IEC-exo treatment alleviated the small mucosal injury after intestinal ischemia following reperfusion. II/R injury is generally accompanied with an enhancement in the levels of TNF- α and endotoxin. In this study, The expression of inflammatory factor such as TNF- α and endotoxin levels in the peripheral blood in the groups treated with IEC-exo were significantly lower than those in the II/R group (Figure 2J and 2K), and the expression of IL-17A mRNA in the small intestinal tissues was also obviously lower in the groups treated with IEC-exo than that in the II/R groups (Figure 2L), which suggesting that IEC-exo treatment decreased the immuno-inflammation responses following II/R insult. These findings suggested that exosomes secreted from IECs exert the protective effects on reducing the damage of IECs. Studies have demonstrated that administration of extracellular vesicles does not cause visible toxicity, immune response and tissue pathological changes up to 3 weeks [25]. In this study, we did not find inflammation and swelling in the organs and weight loss and death of rats after exosome delivery. Therefore, exosomes are not toxic to organs.

Previous studies showed that miRNAs is abundant in the contents of exosomes and exosomes affect target cells via exosomal miRNAs [15]. MiRNAs have been reported to be related to apoptosis, inflammation, autophagy, oxidative stress, and intestinal barrier dysfunction after IIR injury [26–27]. MiRNAs including miR-23a-5p, miR-146a, miR-21, miR-29b-3p, miR-381-p, miR-351-5p, miR-34a-5p, miR-665-3p, miR-682, miR-182 and miR-199a-3p have been involved in modulating mucosal damage following I/R damage in small intestines [27–36]. MiR-23a-5p [27], miR-21[29] miR-182[34] and miR-199a-3p [35] upregulate while miR-29b-3p downregulates[30] in underlying II/R insult. Upregulation of miR-199a-3p dramatically decreased insulin-like growth factor-1, mTOR, and phosphoinositide-3-kinase regulatory subunit 1 protein expression as target genes of miR-199a-3p following II/R damage[35]. MiR-146a is a key regulator of the apoptosis of intestinal epithelial cells during I/R insult through TLR4/NF- κ B pathway [27]. MiR-21[29], miR-29b-3p [30] and miR-682[33] had been demonstrated to exert a protective effect on the gut damage following II/R insult, While miR-23a-5p [26], miR-381-p [31] and miR-351-5p [36] exacerbated II/R injury via various pathways. In this study, primary IEC-18 cells were subjected to 4h OGD and 72 h reoxygenation and miR-23a-3p in IEC-derived exosomes was measured at 72 h after reoxygenation. Our qPCR results validated miR-23a-3p was upregulated in IEC-derived exosomes after OGD. Next, in order to further investigate whether miR-23a-3p alleviates or aggravates the injured gut, MiR-23a-3p plus IEC-derived exosomes were pre-treated with IECs underwent OGD or administrated into the rats by tail vein injection for 3 consecutive days during the II/R development. The results showed that LDH release was significantly lower while cell viability was sharply higher than that in the groups treated with IEC-derived exosomes alone (Figure 2A and 2B). Meanwhile, the damage of the gut and Park-Chiu scores in the groups treated with miR-23a-3p plus IEC-derived exosomes were significantly lower than those in the groups treated with IEC-derived exosomes alone (Figure 4E and 4F), and the expression of

ZO-1 mRNA and protein in the groups treated with miR-23a-3p plus IEC-derived exosomes were obviously higher than those in the groups treated with IEC-derived exosomes alone, suggesting that miR-23a-3p is employed as an anti-inflammatory factor in the injured gut after II/R. However, whether treatment with IEC-derived exosomal miR-23a-3p had an anti-inflammatory effect after II/R insult is unclear. Therefore, we further tested whether knockdown of miR-23a-3p had an opposite result during the II/R development. RNAi packaged with lentivirus was transfected into IEC-18 cells to silence miR-23a-3p. Then, exosomes were collected from the medium and transferred into the rats by tail vein injection for 3 consecutive days after II/R injury. The results showed that the gut damage and Park-Chiu scores in the groups treated with miR-23a-3p knockdown exosomes were significantly higher than those in the groups treated with a control lentivirus vector (Figure 4E and 4F), and the expression of ZO-1 mRNA and protein in the groups treated with miR-23a-3p knockdown exosomes were obviously lower than those in the groups treated with a control lentivirus vector (Figure 4H and 4I), while IL-17A mRNA expression was obviously higher than that in the groups treated with a control lentivirus vector (Figure 4L) accompanied by the increased levels of TNF- α and endotoxin in the peripheral blood (Figure 4J and 4K), which suggested that IEC-derived exosomes with miR-23a-3p knockdown exacerbated intestinal injury after II/R insult.

Moreover, exosomal miRNA regulates its downstream gene expression and the function of target cells. Potential downstream target molecule-MAP4K4 related to miR-23a-3p was selected from TargetScan and miRBase. In this study, it was found that there existed two binding sites between both miR-23a-3p and MAP4K4-3'UTR, which were located in two base fragments of MAP4K4-3'UTR including 508-514 and 566-573 by using Targetscan (www.targetscan.ORG) online analysis software (Figure 5A). The dual luciferase assay indicated that MAP4K4 is the direct downstream target of miR-23a-3p because rno-miR-23a-3p significantly downregulated the expression of luciferase in r-MAP4K4-3'UTR, whereas the downregulation effect disappeared after mutation (Figure 5C). When exosomes derived from miR-23a-3p knockdown lentivirus transfected IEC-18 were administrated into the rats after II/R injury, expression of MAP4K4 in the small intestinal tissues detected by immunofluorescence (Figure 5D and 5E) and Western blot analysis (Figure 5F and 5G) showed was significantly decreased compared with the group treated with a control lentivirus vector whereas PF-6260933, a small-molecule inhibitor of MAP4K4, partly upregulated expression of MAP4K4 meanwhile miR-23a-3p expression in the gut was unchanged compared with the groups treated with miR-23a-3p knockdown exosomes alone (Figure 4A). It was suggesting that exosomal miR-23a-3p directly moderated its downstream target molecule-MAP4K4. MAP4K4, also called hematopoietic progenitor kinase/germinal center kinase-like kinase or Nck-interacting kinase is a serine/threonine kinase belonging to the sterile 20 family of kinases. MAP4K4 is expressed in endothelial cells in the gut and contributes to inflammation by interrupting continuous endothelial cells and tight junctions [37–39]. Studies showed that MAP4K4 inhibition markedly reduces cardiac I/R injury and infarct size in mice [40]. In this study, IEC-derived exosomes were injected into the rats of II/R injury, the gut damage was improved and MAP4K4 expression in the gut was downregulated compared with II/R groups (Figure 4B and 4C). When gut damage became more serious after exosomes derived from miR-23a-3p knockdown lentivirus transfected IEC-18 injection, MAP4K4 expression was upregulated. It was suggesting that expression in the gut was parallel to intestinal injured degree. Finally,

when exosomes derived from miR-23a-3p knockdown lentivirus transfected IEC-18 combined with PF-6260933 were administrated into the rats after II/R injury, the gut damage and Park-Chiu scores were significantly lower compared with those in the groups treated with miR-23a-3p knockdown exosomes alone (Figure 4E and 4F), and the expression of ZO-1 mRNA and protein was obviously higher in the groups treated with miR-23a-3p knockdown exosomes plus PF-6260933 than that in the groups treated with miR-23a-3p knockdown exosomes alone (Figure 4H and 4I) while IL-17A mRNA expression was obviously lower than that in the groups treated with miR-23a-3p knockdown exosomes alone (Figure 4L) accompanied by the decreased levels of TNF- α and endotoxin in the peripheral blood (Figure 4J and 4K), which suggest that inhibition of MAP4K4 activity by PF-6260933 reduced the inflammation and intestinal mucosal injury and counteracts the degree of gut damage exacerbated by IEC-derived exosomes with miR-23a-3p knockdown to some extent after II/R insult.

Conclusions

The study sheds light on the critical role of IEC derived exosomes in alleviating the II/R injury of rats, and this process is mediated by exosomal miR-23a-3p and its target molecule MAP4K4. We may speculate that during the development of II/R injury, exosomes secreted by IECs may be a novel therapeutic approach.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

HY designed the experiment, analyzed the data and drafted the manuscript; SZ designed the experiments and edited the manuscript; JY established the model of II/R rats and contributed to the isolation and identification of exosomes; XZ and CW contributed to the oxygen-glucose deprivation experiments and Immunofluorescence test and Western blot analysis. YW and WC contributed to the mRNA analysis and immunohistology. AW helped to design the experiment.

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Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal experiments were conducted under the rules approved by the Ethics Committee of Children's hospital of Nanjing Medical University (IACUC-2012038).

Consent for publication

All listed authors consent to the submission, and all data are used with the consent of the person generating the data.

Competing Interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The isolation and identification of exosomes from primary intestinal epithelial cell-18 (IEC-18) and detection of miR-23a-3p of exosomes. A. Primary IEC-18 under normal culture conditions (Normal).B.Immunofluorescence staining identification of primary IEC-18 by CK-18 antibody (red fluorescence, ×200).C. Transmission electron microscopy imaging of IEC-derived exosomes. Scale bar =100 nm. D. Nanoparticle tracking analysis of IEC-derived exosomes. E. Expression of exosomal markers ALIX, HSP70, TSG101 and CD63 in IEC-derived exosomes.F. Expression of miRNA-23a-3p in IEC-derived exosomes before and after oxygen-glucose deprivation (OGD).** p<0.01.

Figure 2

Intestinal epithelial cell (IEC)-derived exosomes attenuated the damage of IECs after oxygen-glucose deprivation (OGD) and intestinal ischemia/reperfusion (II/R) injury. A. 1) IECs under normal culture conditions (Normal), 2) IECs underwent OGD treatment (OGD), 3) IECs pretreated with IEC-derived exosomes and exposed to OGD (exo+OGD). Scale bar=50μm. B. Cell viability of IECs treated with or without IEC-derived exosomes after OGD injury. C. LDH release of IECs treated with or without IEC-derived exosomes after OGD injury. D. Morphological changes of the small intestines of the II/R rats treated with PBS, IEC-exo. Scale bar=30cm. E. Histopathological changes of the small intestines of the II/R rats treated with PBS, IEC-exo. Scale bar=100μm. F. Intestinal injury scores shows the histological changes of the

small intestines in the II/R rats treated with PBS, IEC-exo. Immunohistochemical staining of ZO-1 in the small intestinal tissues of the II/R rats treated with PBS, IEC-exo. Scale bar =20 μ m. H-I. Expression of ZO-1 protein (H) and mRNA (I) in the II/R rats treated with PBS, IEC-exo. J-K. TNF- α activity (J), endotoxin concentration (K) in the peripheral blood of the II/R rats treated with PBS, IEC-exo. L. IL-17A mRNA expression of the small intestinal tissues in the II/R rats treated with PBS, IEC-exo. *P <0.05, **P<0.01.

Figure 3

Exosomal miR-23a-3p derived from intestinal epithelial cells (IECs) attenuated the cellular damage after oxygen-glucose deprivation (OGD). A. LDH release of IECs underwent OGD treated with PBS(OGD), IEC-

derived exosomes(OGD+exo), IEC-derived exosomes plus miR-23a-3p (OGD+exo+miR), miR-vector-cn exosomes (OGD+miR-cn-exo), miR-23a-3p knockdown exosomes (OGD+miR-k/d-exo). B. Cell viability of IECs in the groups of OGD),OGD+exo,OGD+exo+miR, OGD+miR-cn-exo, OGD+miR-k/d-exo. C. Immunofluorescence staining of no-load shRNA lentivirus and miR-23a-3p knockdown lentivirus transfected IEC-18. D. Expression of miRNA-23a-3p in the exosomes derived from no-load shRNA lentivirus (miR-cn-exo) or miR-23a-3p knockdown lentivirus transfected IEC-18 (miR23a-3p-k/d-exo). *p<0.05, ** p<0.01.

Figure 4

Exosomal miR-23a-3p derived from intestinal epithelial cells (IECs) decreased gut damage after intestinal ischemia/reperfusion (I/R). A. Expression of miRNA-23a-3p in the small intestinal tissues of I/R rats treated with PBS(I/R), IEC-derived exosomes(I/R+exo), IEC-derived exosomes plus miR-23a-3p (I/R+exo+miR), miR-vector-cn exosomes (I/R+miR-cn-exo), miR-23a-3p knockdown exosomes (I/R+miR-k/d-exo), miR-23a-3p knockdown exosomes plus PF6260933 (I/R+miR-k/d-exo+PF 6260933). B- C. Immunoblotting (B) and western blot analysis (C) of MAP4K4 of the small intestinal tissues in the groups of I/R, I/R+exo, I/R+exo+miR, I/R+miR-cn-exo, I/R+miR-k/d-exo, I/R+miR-k/d-exo+PF6260933. D. Morphological changes of the small intestines in the groups of I/R, I/R+exo, I/R+exo+miR, I/R+miR-cn-exo, I/R+miR-k/d-exo, I/R+miR-k/d-exo+PF 6260933. Scale bar=30cm. E-F. Histopathological changes (E) and intestinal injury scores (F) of the small intestines in the groups of I/R, I/R+exo, I/R+exo+miR, I/R+miR-cn-exo, I/R+miR-k/d-exo, I/R+miR-k/d-exo+PF 6260933. Scale bar =100µm. G. Immunohistochemical staining of ZO-1 in the small intestinal tissues in the groups of I/R, I/R+exo, I/R+exo+miR, I/R+miR-cn-exo, I/R+miR-k/d-exo, I/R+miR-k/d-exo+PF6260933. Scale bar =20µm. H-I. Expression of ZO-1 protein (H) and mRNA (I) in the groups of I/R, I/R+exo, I/R+exo+miR, I/R+miR-cn-exo, I/R+miR-k/d-exo, I/R+miR-k/d-exo+PF6260933. J-K. TNF-α activity (J), endotoxin concentration (K) in the groups of I/R, I/R+exo, I/R+exo+miR, I/R+miR-cn-exo, I/R+miR-k/d-exo, I/R+miR-k/d-exo+PF6260933. L. IL-17A mRNA expression of the small intestinal tissues in the groups of I/R, I/R+exo, I/R+exo+miR, I/R+miR-cn-exo, I/R+miR-k/d-exo, I/R+miR-k/d-exo+PF6260933. *p<0.05, ** p<0.01.

Figure 5

Regulating effect of exosomal miR-23a-3p derived from intestinal epithelial cells (IECs) on its downstream target MAP4K4. A. Expression of putative miR-23a-3p targets MAP4K4. B, D. Immunoblotting (B) and western blot analysis (D) of MAP4K4 of the small intestinal tissues in the groups of II/R+miR-cn-exo, II/R+miR-k/d-exo, II/R+miR-k/d-exo+PF6260933. C. The detection of dual luciferase reporter gene showed that miR-23a-3p inhibited MAP4K4 activity. Rluc: sea kidney luciferase, Fluc: firefly luciferase. E-F.

Immunofluorescence staining (E) and mean optical density (MOD) (F) of MAP4K4 in the small intestines of II/R rats treated with miR-cn-exo, miR-23a-3p-k/d-exo and miR-23a-3p-k/d-exo plus PF-6260933.* $p < 0.05$, ** $p < 0.01$.