

Exosomal miR-451 from human umbilical cord mesenchymal stem cells attenuates burn-induced acute lung injury

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Abstract

Background: The aim of this study was to investigate the molecular mechanism of human umbilical cord mesenchymal stem cells (MSCs)-derived exosomes (hUCMSC-Exos) in regulating burn-induced acute lung injury (ALI).

Methods: In this study, we initially isolated exosomes from hUCMSCs and identified them by transmission electron microscopy. The expression of the protein markers CD9 and CD63 in the exosomes was determined by western blot analysis. The expression of miR-451 in the hUCMSC-Exos was determined by qRT-PCR. The levels of TNF- α , IL-1 β , and IL-6 in lung tissues and serum as well as the levels of malondialdehyde, myeloperoxidase, superoxide dismutase in lung tissues were detected by ELISA. Hematoxylin-eosin stain was used to observe the morphological changes of lung tissues after burn. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assays were performed to detect apoptosis in lung tissues after burn. The expression of proteins related to the Toll-like receptor 4 (TLR4)/NF- κ B signaling pathway in lung tissues after burn was analyzed by western blotting.

Results: Our results showed that hUCMSC-Exos successfully decreased TNF- α , IL-1 β , and IL-6 levels in rats after burn, and this reduction was reversed when the miR-451 expression in the hUCMSC-Exo group was inhibited. hUCMSC-Exo-derived miR-451 improves ALI via the TLR4/NF- κ B pathway.

Conclusion: We demonstrated that exosomes derived from hUCMSCs mediate miR-451 to attenuate burn-induced ALI.

Keywords: Acute lung injury; hUCMSC exosomes; miR-451

1. INTRODUCTION

Critical burns requiring emergency surgery are often accompanied by damage to multiple organs. Acute lung injury (ALI) is one of the most common complications, often presenting in the early stage of severe burns. Once ALI occurs, it is extremely easy for patients to develop acute respiratory distress syndrome (ARDS), which is responsible for the high mortality of burn patients.¹ To the best of our knowledge, at present, symptomatic therapy is the main clinical treatment for ALI induced by severe burn. There is a lack of effective treatment measures to prevent and alleviate the occurrence of ALI after burn.² Thermal scald, the main type of burn, is characterized by a cascade of inflammatory responses both locally and systemically.³ Some pathways related to inflammatory responses, such as Toll-like

receptor 4 (TLR4)/nuclear factor kappa enhancer binding protein (NF- κ B),⁴ nuclear factor-erythroid 2-related factor-2 (Nrf2)/haem oxygenase-1 (HO-1),⁵ and mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinases (ERK),⁶ are involved in ALI development.

Mesenchymal stem cells (MSCs) constitute a promising source of cells for cell-based therapeutic strategies. Increasing studies have reported that human umbilical cord mesenchymal stem cells (hUCMSCs) can be applied to ameliorate inflammatory conditions, such as spinal cord injury,⁷ Alzheimer's disease,⁸ and hyperglycemia-induced retinal inflammation.⁹ Recently, an increasing number of studies have revealed that exosomes, extracellular vesicles released by MSCs, offer therapeutic benefits.¹⁰ Exosomes were described as vesicles 30 to 120 nm in diameter.^{11,12} MSC-derived exosomes have been recognized as very attractive candidates for the treatment of lung diseases, including ALI.¹³ Li et al revealed that MSC exosomes can alleviate endotoxin-induced ALI by suppressing inflammatory cell infiltration and inflammatory response.¹⁴ Porzionato and coworkers recently determined that MSC-derived extracellular vesicles reduced lung injury in a rat model of bronchopulmonary dysplasia.¹⁵

MSC-derived exosomes carry diverse cargoes of protein, mRNA, and miRNA¹⁶ and transfer them to recipient cells to regulate cellular processes. Accumulating evidence suggests that a miRNA transferred by exosomes could mediate an inflammation reaction through the regulation of target proteins in an

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inflammatory signaling pathway. A report has demonstrated that miR-451 was significantly upregulated in MSC exosomes,¹⁷ and miR-451 has been shown to target TLR4 to inhibit the TLR4/NF- κ B signaling pathway.¹⁸ Hence, we speculated that exosomes derived from hUCMSCs (hUCMSC-Exos) modulate miR-451 to ameliorate inflammation via the TLR4/NF- κ B and thus improve tissue health after burn-induced ALI.

2. METHODS

2.1. Cell culture and transfection

The hUCMSCs were obtained from the First Affiliated Hospital of Anhui Medical University and maintained in Dulbecco's modified Eagle's medium (DMEM)/high glucose medium (HyClone Laboratories, Logan, UT, USA) supplemented with 10% defined fetal bovine serum (HyClone Laboratories) at 37°C in 5% CO₂.

The hUCMSCs were transfected with a miRNA inhibitor control or a miR-451 inhibitor using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.2. Isolation and characterization of hUCMSC exosomes

The exosomes were isolated and purified from the supernatant of the hUCMSCs. Briefly, 10% FBS DMEM was replaced with 10% exosome-free FBS DMEM when the cultured hUCMSC had reached 80% to 90% confluence. The exosome-free FBS was obtained by subjecting the FBS to ultracentrifugation at 100 000g for 16 hours. The conditioned medium used for the hUCMSC was centrifuged at 300g for 20 minutes, 2000g for 20 minutes, and 10 000g for 30 minutes to remove cell debris. The cleared supernatant was transferred to a new glass tube and kept on ice. The supernatant was mixed with ExoQuick-TC exosome precipitation solution (System Biosciences, Mountain View, CA, USA) (5:1) in a new tube and incubated at 4°C overnight. Then, the mixture was centrifuged at 1500g for 30 minutes, and the supernatants were discarded. After centrifugation, the hUCMSC-exosomes were dissolved in 100 to 200 μ L of phosphate buffer saline (PBS) and then stored at -80°C until further use. The total protein content of the exosomes was also quantified. To avoid the influence of substances in ExoQuick-TC, we used ExoQuick-TC with fresh conditioned medium in our test with the same protocol, adding the same quantities of PBS and then storing the cells with exosome miRNA at -80°C until further use.

Nanosizer technology (Malvern Instruments, Malvern, Worcestershire, UK) was adopted to evaluate the size distribution of the hUCMSC-Exos. The purified exosomes were resuspended in PBS, and the morphology of the exosomes was observed under a transmission electron microscope. The expression of the specific exosome markers CD9 and CD63 was detected by western blotting.

2.3. QRT-PCR

The miR-451 was extracted using the miRVana extraction kit (Ambion, Austin, TX, USA). For miR-451 quantification, 10 ng of total RNA was reverse transcribed and amplified using a miRNA reverse transcription and detection kit (Applied Biosystems, Carlsbad, CA, USA). All results were normalized to U6 levels, which were determined by the ABI miRNA U6 assay kit (Applied Biosystems).

2.4. Western blotting

Proteins were extracted with RIPA buffer. The protein concentration was determined using a protein assay kit (Bio-Rad). Approximately 30 μ g of protein from each sample was separated on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk and incubated with anti-CD9 (1:2000, Abcam, Cambridge, UK), anti-CD63 (1:1000, Abcam), anti-p65

(1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TLR4 (1:500, Santa Cruz Biotechnology), and anti-p-p65 (1:1000, Santa Cruz Biotechnology) antibodies overnight at 4°C, followed by incubation with the corresponding secondary antibodies for 1 hour at room temperature. The proteins were detected on membranes, after washing in TBST, using Super ECL Plus detection reagent (Thermo Fisher Scientific, Carlsbad, CA, USA).

2.5. ELISA

The levels of TNF- α , IL-1 β , and IL-6 in lung tissues and serum from rats as well as the levels of myeloperoxidase (MPO), malondialdehyde (MDA), and superoxide dismutase (SOD) in lung tissues were determined using commercial ELISA kits (Abcam) according to the manufacturer's instructions. The concentrations were calculated according to the corresponding OD value.

2.6. HE staining

The lung tissues were embedded in paraffin after fixation with 4% paraformaldehyde. Preparative sections were stained with hematoxylin-eosin (HE) stain for light microscopy.

2.7. TUNEL assay

To detect cell apoptosis in the lung tissues, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was performed according to the manufacturer's instructions. Image-Pro Plus software (Media Cybernetics, MD, USA) quantified apoptosis by counting the number of TUNEL-positive cells.

2.8. Severe burn rat model and administration of hUCMSC-exosomes

This project was approved by the Institutional Animal Care and Use Committees of the First Affiliated Hospital of Anhui Medical University. Six-weeks-old female SD rats (200 \pm 20g) were raised at the Shanghai Laboratory Animals Center (SLAC; Shanghai, China). They were housed under standardized conditions with controlled temperature and humidity and a 12/12-hour day/night light cycle. Rats were given free access to diet and water. To generate the severe burn rat model, a rat model with a third degree burn on 30% of the total body surface area (TBSA) and a full-thickness burn wound was established as described previously. Briefly, rats were anaesthetized by intraperitoneal injection of 300 mg/kg Avertin (20 mg/mL) (2,2,2-tribromoethanol, Sigma, USA), and the dorsal hair was completely removed. The backside of the rats was placed in hot water (94°C) for 12 seconds, which caused a 30% TBSA full-thickness burn. The wound was treated with a 1% tincture of iodine and kept dry to prevent infection. A balanced salt solution (5 mL) was immediately administered to prevent shock.

Burned rats were randomly assigned to different treatment groups via tail vein injection: hUCMSC-Exo group, injection with 800 μ g (RNA concentration) hUCMSC-exosomes suspended in 1 mL of PBS; NCI-Exo group, 800 μ g hUCMSC-exosomes transfected with the inhibitor control suspended in 1 mL of PBS; and miR-451i-Exo group, 800 μ g hUCMSC-exosomes transfected with miR-451 inhibitor suspended in 1 mL of PBS. Rats in the PBS group were given an equal volume of PBS, and rats in the sham group were placed in water at 37°C for 12 seconds and subjected to the same processes as those applied to the burned rats. Wounds were left open, and animals were sacrificed at 24 or 48 hours after treatment. Lung tissue samples were collected for the pathology analysis.

2.9. Statistics

All data are shown as the mean and SD. Differences between means were determined by Student's *t* test with two groups or ANOVA if more than two groups were compared, and *p* < 0.05 was considered statistically significant.

3. RESULTS

3.1. Characterization of the hUCMSC-Exos

We initially isolated exosomes from the culture supernatants of the hUCMSCs. Transmission electron microscopic observation of the hUCMSC-Exos revealed the presence of spherical vesicles, with a typical cup shape and a diameter that ranged from 40 to 160 nm (Fig. 1A, B). To examine whether the exosomes had been successfully purified, we performed western blot analysis, and the isolated hUCMSC-Exos were found to express high levels of CD9 and CD63 (Fig. 1C). Furthermore, miR-451 was significantly upregulated in the hUCMSC-Exos relative to the hUCMSCs (Fig. 1D). Taken together, the results indicated that the isolated hUCMSC-Exo fraction was pure and could be used for subsequent experiments.

3.2. hUCMSC-Exo-derived miR-451 suppresses burn injury-induced inflammation

To investigate the effect of hUCMSC-Exos on inflammatory cytokines in burned rats, the rats were randomly divided into six groups: sham group, burn group, burn + PBS group, burn + Exo group, burn + NCI-Exo group and burn + miR-451i-Exo group. As presented in Fig. 2A, a pronounced reduction in miR-451 expression was observed in exosomes extracted from the hUCMSCs transfected with miR-451 inhibitor. We examined the inflammatory factors present in the rat lung tissues using ELISA and found that at 6, 12, 24, and 48 hours after burn injury, the TNF- α , IL-1 β , and IL-6 levels were remarkably increased compared to those of the sham group. Exposure to hUCMSC-Exos successfully decreased the levels of these factors, an outcome that was reversed when miR-451 expression in hUCMSC-Exo group was inhibited (Fig. 2B–D, Supplementary Fig. 1, <http://links.lww.com/JCMA/A30>). In a concordant finding, a similar pattern in the levels of TNF- α (Fig. 2E), IL-1 β (Fig. 2F), and IL-6 (Fig. 2G) was observed in the rat serum. The results showed

that hUCMSC-Exo-derived miR-451 suppressed burn injury-induced inflammation.

3.3. hUCMSC-Exo-derived miR-451 improves ALI via the TLR4/NF- κ B pathway

We further investigated the effect of hUCMSC-Exo-derived miR-451 on ALI in the burned rats. After 6 hours of ALI and exosome treatment, we measured the lung functional assays, including inspiratory capacity (IC, ml), central airway resistance (Rn, cmH₂O/s mL) and lung compliance (Crs, mL/cmH₂O), as presented in Table. The ELISA results revealed that at both 24 and 48 hours after burn, MDA, MPO, and SOD levels in the burn models were markedly lower than those in the sham group; that is, administration of hUCMSC-Exos significantly elevated the levels compared to the PBS-treated group, and these induced effects could be reversed by miR-451 inhibition (Fig. 3A–C). Histological evaluation of the cutaneous wounds collected from the different groups at 48 hours revealed that administration of hUCMSC-Exos significantly suppressed lung injury, an outcome that was reversed after the miR-451 was inhibited (Fig. 3D). TUNEL assays were performed to detect apoptosis in lung tissues isolated 48 hours after burn. As indicated in Fig. 3E, the ability of hUCMSC-Exos to suppress apoptosis was markedly compromised when the exosome miR-451 expression was inhibited. Using qRT-PCR, we found that miR-451 was remarkably upregulated in the group administered hUCMSC-Exos relative to the group administered PBS (Fig. 3F). To gain further insight into the underlying mechanism by which the hUCMSC-Exos-derived miR-451 improved ALI, we detected the expression of the TLR4/NF- κ B signaling pathway, which is one of the most important regulators of the inflammatory reaction. We examined TLR4 expression and its downstream target proteins NF- κ B/P65 and p-P65. Compared with those of the sham group, the TLR4 and p-P65 protein levels were significantly increased in the burned rats, and administration of the hUCMSC-Exos significantly downregulated TLR4

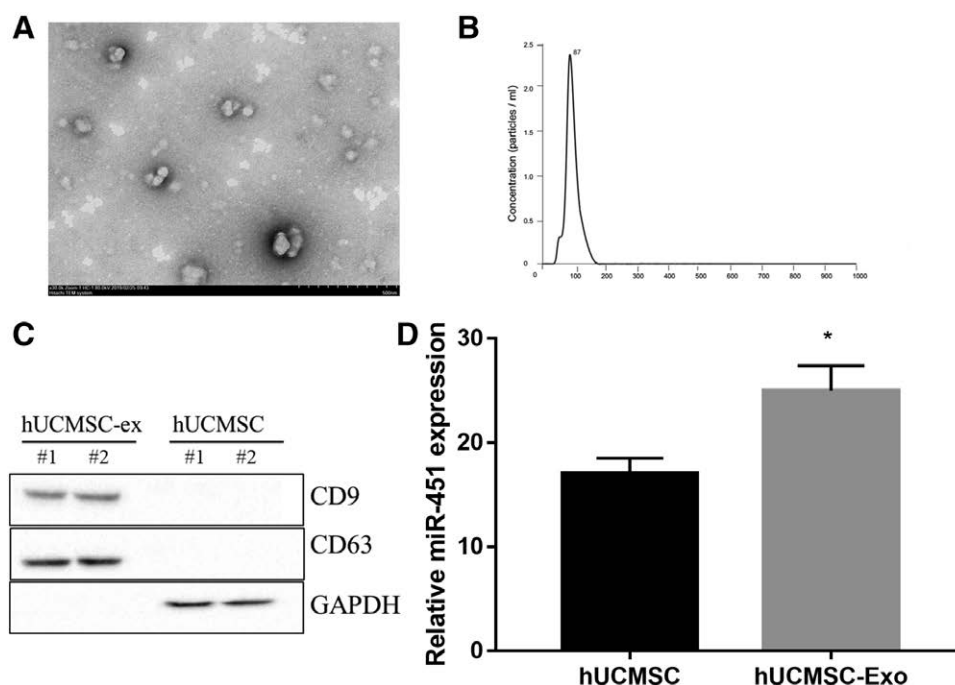


Fig. 1 Characterization of hUCMSC-Exos. A, The ultrastructure of hUCMSC-Exo was analyzed by transmission electron microscopy. Scale bar = 500 nm. B, Diameter ranges of hUCMSC-Exo under transmission electron microscopy. C, Expression of the protein markers CD9 and CD63, which are commonly found in exosomes, was determined by western blot. D, MiR-451 expression in hUCMSCs and hUCMSC-Exo cells was examined by qRT-PCR (* $p < 0.05$ vs hUCMSC). hUCMSC, human umbilical cord mesenchymal stem cell.

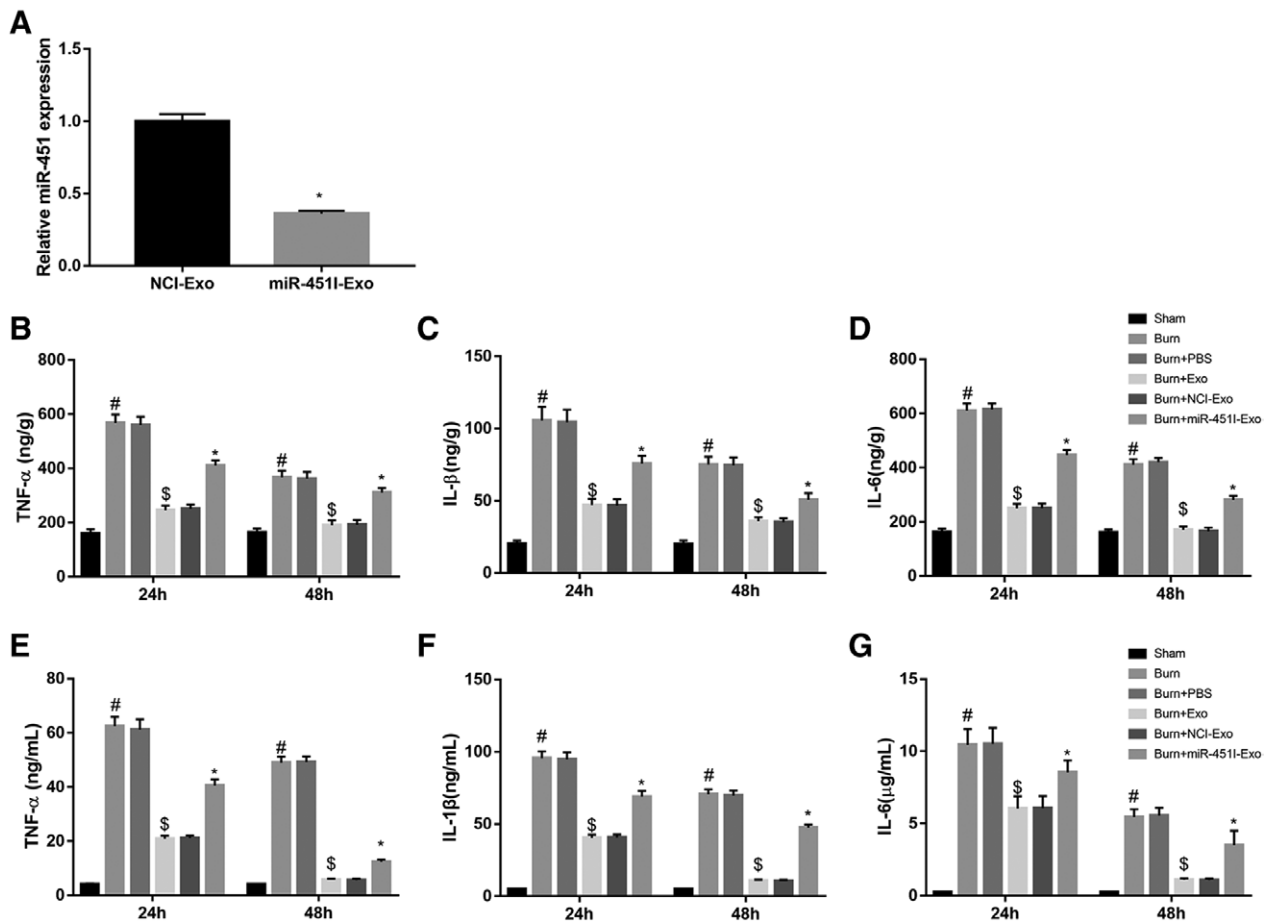


Fig. 2 hUCMSC-Exo-derived miR-451 suppresses burn injury-induced inflammation. Rats were randomly divided into six groups: sham group, burn group, burn + PBS group, burn + NCI-Exo group (exosomes were extracted from hUCMSCs transfected with miRNA inhibitor control) and burn + miR-451-Exo group (exosomes were extracted from hUCMSCs transfected with miR-451 inhibitor). A, MiR-451 expression in hUCMSC-Exo from each group was determined by qRT-PCR. B–D, The levels of TNF- α , IL-1 β , and IL-6 in lung tissues isolated at 24 and 48 h after burn were detected by ELISA. E–G, The levels of TNF- α , IL-1 β , and IL-6 in serum isolated at 24 and 48 h after burn were detected by ELISA (# p < 0.05 vs sham; \$ p < 0.05 vs PBS; * p < 0.05 vs NCI-Exo). hUCMSC, human umbilical cord mesenchymal stem cell; PBS, phosphate buffer saline.

Table

Lung function indexes of rats in each group of the ALI model at 6h (mean \pm SD, n = 10)

Group	IC, ml	Rn, cmH ₂ O/s-ml	Crs, ml/cmH ₂ O
Sham	8.60 \pm 0.31	0.074 \pm 0.006	0.42 \pm 0.03
Burn	7.40 \pm 0.63*	0.108 \pm 0.015*	0.33 \pm 0.05*
Burn + PBS	7.37 \pm 0.59	0.111 \pm 0.012	0.32 \pm 0.04
Burn + Exo	8.15 \pm 0.60**	0.092 \pm 0.014**	0.38 \pm 0.02**
Burn + NCI-Exo	8.21 \pm 0.58	0.089 \pm 0.017	0.39 \pm 0.02
Burn + miR-451-Exo	7.68 \pm 0.35***	0.105 \pm 0.016***	0.35 \pm 0.03***

(* p < 0.05 vs sham, ** p < 0.05 vs Burn + PBS, *** p < 0.05 vs Burn + NCI-Exo).

ALI = acute lung injury; Crs = lung compliance; Exo = exosome; IC = inspiratory capacity; NCI-Exo = exosome extracted from hUCMSCs and transfected with a miRNA inhibitor control; PBS = phosphate buffer saline; Rn = central airway resistance; 451-Exo = exosome extracted from hUCMSCs and transfected with a miR-451 inhibitor.

and p-P65 protein expression at 48 hours, an outcome that was reversed when miR-451 expression in the hUCMSC-Exo group was inhibited. No statistically significant difference was observed in P65 expression among the groups after burn injury (Fig. 3G). From these results, it is clear that hUCMSC-Exo-derived miR-451 improved ALI via the TLR4/NF- κ B pathway.

4. DISCUSSION

Exosomes derived from MSCs were previously shown to be effective in diverse diseases.¹⁹ MSC exosome-treated defects displayed a concomitant reduction in the proinflammatory synovial cytokines IL-1 β and TNF- α in cartilage repair.²⁰ The expression of proinflammatory cytokines such as TNF- α , IL-1 β , and IL-6 was significantly decreased in hUCMSC exosome-treated mice with inflammatory bowel disease.²¹ The results of Li et al indicated that hUCMSC exosomes suppressed LPS-induced macrophage inflammation and weakened LPS-induced ALI in rats.²² In this study, we successfully isolated and characterized exosomes from hUCMSCs. In accordance with the above reports, we demonstrated that administration of hUCMSC-Exos reduced the levels of TNF- α , IL-1 β , and IL-6 in lung tissues after burn and suppressed burn injury-induced inflammation.

Exosome-mediated miRNA transfer plays a pivotal role in regulating inflammation.²³ In monocytes, miRNA-122 transferred via exosomes restricted the HO-1 pathway and elevated levels of proinflammatory cytokines in mice with alcoholic hepatitis.²⁴ A recent study showed that activated macrophages secrete miR-155-enriched exosomes and identified macrophage-derived miR-155 as a paracrine regulator for inflammation.²⁵ Increased miR-124-3p in microglial exosomes

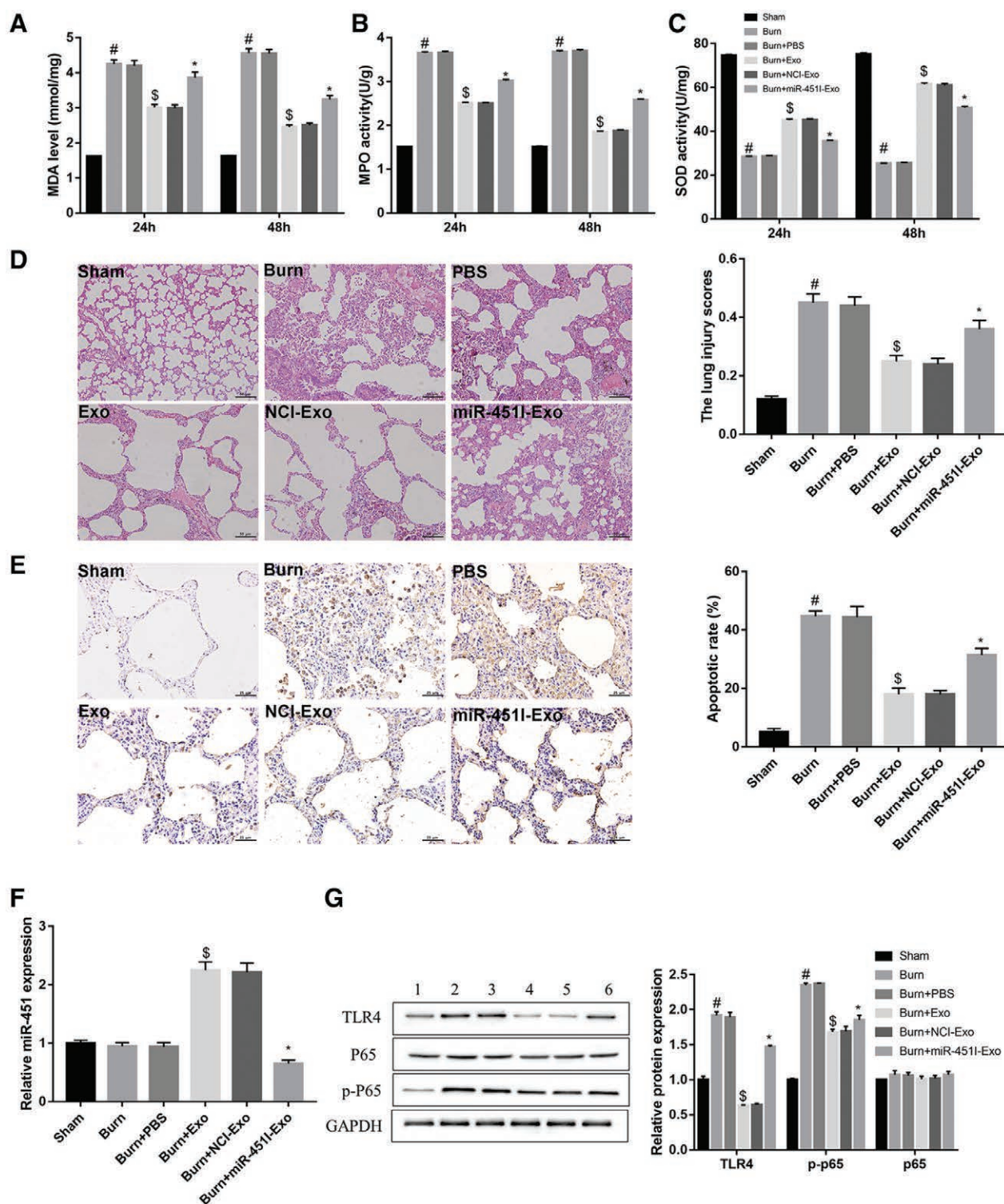


Fig. 3 hUCMSC-Exo-derived miR-451 improves acute lung injury in rats with burn injury via the TLR4/NF- κ B pathway. Rats were randomly divided into six groups: sham group, burn group, burn + PBS group, burn + NCI-Exo group (exosomes were extracted from hUCMSCs transfected with miRNA inhibitor control) and burn + miR-451-Exo group (exosomes were extracted from hUCMSCs transfected with miR-451 inhibitor). A–C, The levels of MDA, MPO, and SOD in lung tissues isolated at 24 and 48h after burn were detected by ELISA. D, HE stain was used to observe the morphological changes of lung tissues isolated at 48h after burn. E, TUNEL was performed to detect apoptosis in lung tissues isolated 48h after burn. F: MiR-451 expression in lung tissues isolated 48h after burn was determined by qRT-PCR. G: The expression of proteins related to TLR4/NF- κ B signaling pathway in lung tissues isolated at 48h after burn were analyzed by western blot, $n = 10$. (* $p < 0.05$ vs sham; \$ $p < 0.05$ vs PBS; * $p < 0.05$ vs NCI-Exo). HE, hematoxylin–eosin; hUCMSC, human umbilical cord mesenchymal stem cell; MDA, malondialdehyde; MPO, myeloperoxidase; PBS, phosphate buffer saline; SOD, superoxide dismutase; TLR4, Toll-like receptor 4.

following traumatic brain injury represses neuronal inflammation.²⁶ In addition, miR-181c blocks TLR4 expression by directly binding to its 3'-UTR to limit the inflammatory reaction.²⁷ Our study demonstrated a significant inflammation-reducing effect of hUCMSC-Exo administration on severe burn-induced ALI, which was reversed when miR-451 expression in hUCMSC-Exos was inhibited.

TLR4 signaling has a pivotal role in inflammatory processes.^{28–30} Recent studies have shown that TLR4-NF- κ B-mediated inflammation is involved in a range of conditions, such as in the retina after optic nerve crush,³¹ atherosclerosis,³² and traumatic brain injury.³³ Mechanistic analysis confirmed that miR-451 upregulation inhibited LPS-induced TLR4 expression, which was identified as a direct target of miR-451.¹⁸ To further investigate the mechanism contributing to the process of miR-451-mediated inflammation, we examined TLR4 expression and its downstream target proteins NF- κ B/P65 and p-P65 in vivo. Notably, hUCMSC-Exo administration significantly downregulated TLR4 and p-P65 protein expression, which was reversed by miR-451 inhibition. Thus, these findings reveal that hUCMSC-Exo-derived miR-451 reduces the inflammation caused by ALI and that it affects the TLR4/NF- κ B pathway.

In conclusion, our findings demonstrate that hUCMSC-Exos can effectively improve burn-induced inflammation. Further data elucidated the importance of miR-451 in the process of hUCMSC-Exos-dependent regulation of inflammation. The miR-451 inhibitor increased TLR4 expression and subsequently induced p65 activation in rats with burn-induced ALI. These findings might provide clues for a better understanding of the mechanisms of ALI and provide a potential target for clinical therapy for ALI.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://doi.org/10.1016/j.jcma.2018.01.013>.

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