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Mammary epithelial cell-derived exosomal miR-155-inhibitor played a key role in the treatment of mastitis via down-regulation of TLRs/NF-κB signaling pathway to inhibit inflammatory response

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ABSTRACT

Mastitis is a common disorder in women capable of altering the normal physiological function of the mammary gland. It has been reported that mammary epithelial cells (MECs) could be involved in treating mastitis by regulating the inflammatory response and miR-155 might participate in this process. However, the effects of MECs-derived exosomal miR-155-inhibitor in treating mastitis and the regarding mechanism are still unknown. In our study, mouse mammary epithelial cells (HC11) were applied to study the role of MECs-derived exosomal miR-155-inhibitor in the treatment of mastitis and explore the mechanism. Results in our study showed that specific markers including CD63 and Apo-A1 were expressed in blank exosomes and exosomes containing miR-155-inhibitor isolated from transfected HC11 cells. Results of immunofluorescence showed that the blank exosomes and exosomes (containing miR-155-inhibitor) labeled with PKH26 were absorbed in HC11 cells. The level of miR-155 was decreased obviously in Engineered exosomes with miR-155-inhibitor and HC11 cells Transfected with exosome containing miR-155-inhibitor. The level of miR-155 was increased and cell apoptosis was promoted obviously in HC11 cells induced by LPS, however, they were decreased obviously after transfecting with an exosome containing miR-155-inhibitor. The level of TLR2, TLR4, TLR6, NF-κB, TNF-α, and IL-1β was increased obviously in LPS-induced HC11 cells, however, they were decreased obviously after transfecting with an exosome containing miR-155-inhibitor. The change in IL-10 level is opposite to the above genes. Taken together, exosomal miR-155-inhibitor could decrease the apoptosis of MECs and inhibit the inflammatory response to treat mastitis by down-regulation in the TLRs/NF-κB signaling pathway, which might be a new therapeutic target for mastitis

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Introduction

Cow mastitis is a common disease that plagues dairy cattle husbandry and causes severe economic losses (1). Mastitis can be induced by physical irritation, microcirculation disturbance, and infection by pathogenic microorganisms, which are the main pathogenic factors (2). Invasion of pathogenic bacteria into the host is a complex, dynamic, and multifactorial action process, and an in-depth study of how pathogenic bacteria invade host cells is a key link to uncovering the pathogenic mechanism of mastitis.

MicroRNAs (miRNAs) are small RNA molecules, which consist of 21-23 bases with a high degree of conservation (3). MiRNAs are involved in regulating target gene expression by degrading or inhibiting mRNAs after specifically binding to the target genes (4). Studies have reported that the expression level of some miRNAs changes rapidly after infection by pathogenic microorganisms, and miRNAs can be used as biological markers for disease diagnosis and regulation (5). The changes of miRNAs in the occurrence of inflammation are closely related to the TLRs signaling pathway (6). Infection of mammary tissue by pathogenic microorganisms can cause inflammatory cytokines and ROS release for phagocytic elimination of pathogenic microorganisms (7). This effect is regulated by the same TLRs pathway, which in turn has a close interaction with miRNAs. Therefore, an in-depth study of the interactive relationship between miRNAs and TLRs to identify the key targets of their action and to regulate them has profound implications for the prevention and control of mastitis (8).

MiR-155 can participate in the occurrence and development of the immune response responses (9). MiR-155 has several target genes, and a previous study reported that miR-155 can directly act on the TLR4 signaling pathway via AP1, NF-κB regulation of innate immune responses (10). Targeted regulation of the TLRs signaling pathway by miR-155 is involved in various pathophysiological processes in cells, and it has an extremely broad prospect of clinical application (11). Some studies have confirmed that appropriate supplementation of selenium can regulate the body's immune function of mice through miR-155 and alleviate inflammatory injury in the mammary gland (8). Thus, miR-155 regulates the TLRs/NF-κB signaling

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pathway or would be a key contributing pathway to the pathogenesis of mastitis.

Extracellular vesicles refer to cell-derived vesicular bodies, in which exosomes are derived from multivesicular bodies, whereas microvesicles, ribosomes, and apoptotic bodies are generated by shedding from the membrane of the cells (12). Extracellular vesicles carry various transport substances, including RNA, proteins, lipids, and DNA, which can be taken up by other cells and elicit various phenotypic responses (13). However, the fusion of exosomes and cells is not random, with some molecules in the exosomes helping target specific tissues, while others ensure that the possibility of non-specific binding is reduced (14). Exosomes can penetrate tissues, enter the blood, and even stride across the blood-brain barrier (15). After exosomes enter the systemic circulation, immune cells as well as excretory organs such as the liver, lung, and kidney must be avoided (16). Their target tissue efficiency depends on the degree of functionalization as well as the strength of interaction with the target cells. Based on the molecular transport capacity of exosomes as well as targeting properties, specific cell-targeted delivery vehicles have been produced (17). Specific protein or RNA molecules can also be loaded into exosomes to make them targetable by imparting their cell and tissue specificity through modification of exosome surface molecules (18). The engineering properties of exosomes endow exosomes with new features. Engineering exosomes encapsulating miR-155 for surface molecular modification to concentrate them toward mammary epithelial cells to specifically repair the cellular immune response after mastitis would greatly enhance the therapeutic efficacy of targeting microRNAs.

In this study, we aimed to use engineered exosomes to encapsulate miR-155 for its targeted delivery to murine mammary epithelial cells, improve the therapeutic efficiency of mastitis, and explore the role of miR-155 in improving the immune inflammatory reaction after mastitis via TLRs/NF-κB signaling pathways and the regarding mechanisms.

Materials and Methods

Regents

ELISA Kits for the detection of TNF-α, IL-1β, and IL-10 were purchased from Beyotime (Beyotime Biotechnology, Beijing, China). Specific antibodies against CD63, Apo-A1, PKH26, Bax, Bcl-2, TLR2, TLR4, TLR6, NF-κB, and β-action were obtained from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and treatment

HC11 cells were obtained from the American Type Culture Collection (ATCC). Briefly, HC11 cells were cultured by using RPMI-1640 medium (containing 100 mug/mL-streptomycin, 100 U/mL-penicillin, and 10%-fetal bovine serum (FBS)) at 37℃ in 5% CO_2.

For the exosome treatment, HC11 cells were cultured with 20 μg/mL of HC11-derived exosomes with or without miR-155 inhibitor and control medium in 6-well plates for 24 h for immunofluorescence, q-PCR, and Western blot (WB). Then, HC11 cells were exposed to LPS at the dose of 1000 ng/mL in a complete medium (exosome-free FBS) to establish the inflammatory *in vitro* model. After treatment for 24 h with LPS, the supernatant from the dish was discarded, and a fresh medium without FBS was added. The LPS-stimulated HC11 cells were cultured with 20 μg/mL of HC11-derived exosomes with or without miR-155 inhibitor and control medium in 6-well plates for 24 h for TUNEL assay, Western blot, real-time PCR, and ELISA experiments.

Exosome isolation and identification

The isolation of exosomes from the supernatant of HC11 cells was conducted by ultracentrifugation. In brief, the medium was centrifuged (1000g, 10 min) to precipitate cells. Then, cellular debris was removed by centrifugation (12,000g, 30 min). Finally, exosomes were obtained from the supernatant by centrifugation (100,000g, 60 min) at 4℃. Exosomes were washed 3 times in 5mL PBS and then re-suspended with 100 μL PBS for further identification. The concentration of total proteins in exosomes was determined by using a Pierce BCA kit ((Pierce Company, USA). Exosomal markers including CD63 and Apo-A1 were detected by WB. Exosomes' size and quantity were quantified by using the Malvern NanoSight NS300 particle size analysis (NTA) (NanoSight Ltd, UK).

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to observe exosomes. Briefly, exosomes were resuspended and applied to copper grids for 10-30 s. Coverslips were mounted with drops of 2% phosphotungstic acid, pH 7.0, on the same slide for 5 s. After 20 minutes, the grid was dry and was observed by using a transmission electron microscope (Hitachi, Tokyo, Japan).

Exosome labeling and tracking

PKH26 red fluorescent dye (Sigma-Aldrich, USA) was used to label exosomes according to the manufacturer's instructions. The nucleus was stained with DAPI. Then, an exosome absorption study was conducted by resuspending the labeled exosomes and adding them to the unstained macrophages. After incubation at 37 ℃ for 12 h, the cell staining was observed by using a fluorescence microscope (IX83, Olympus, Tokyo, Japan).

Transfection

HC11 cells or LPS-stimulated HC11 cells were transfected with miR-155 inhibitor by using Lipofectamine 3000 (Thermofisher, USA). The sequence of the miR-155 inhibitor is 5′-UCUACUCUUUCUAGGAGGUUGUGA-3′.

TUNEL assay

TUNEL staining in vitro was performed by using a commercially available kit (Abcam, Shanghai, China). The stained cells were observed by using a fluorescence microscope (Olympus, Japan) to quantify apoptotic cells. The mean number of TUNEL-positive cells was counted in 10 random regions by two independent handlers.

Measurement of TNF-α, IL-1β, and IL-10 by ELISA

The concentration of TNF-α, IL-1β, and IL-10 in HC11 cells was measured using commercialized ELISA kits. A microplate reader (BMG LABTECH, Offenburg, Germany) was applied to determine the absorbance.

qRT-PCR

TRIzol reagent (T9424, Sigma-Aldrich, Beijing, China) was applied to extract RNA from cells. cDNA synthesis was performed using a Reverse Transcription Kit (RR037Q, Takara, Dalian, China). The PCR reaction system (25 μL) comprises 0.3 μM primers for each, 12.5 μL Maxima SYBR Green qPCR Master Mix (A46012, Invitrogen, ThermoFisher Scientific), and 2.5 μL cDNA. Primers were as follows: miR-155(mouse): 5′-CTAGCTAGCGAACTATGAACCGTGGCTG-3′ and 5′-CCGCTCGAGGAGATGTTGTTTAGGATACTGC-3′, TLR2 (mouse): 5′-CAGACGTAGTGAGCGAGCTG-3′ and 5′-GGCATCGGATGAAAAGTGTT-3′, TLR4 (mouse): 5′-GCTTTCACCTCTGCCTTCAC-3′ and 5′-CGAGGCTTTTCCATC CAATA-3′, TLR6 (mouse): 5′-GCAACATGAGCCAAGACAGA-3′ and 5′-GTTTTGCAACCGATTGTGTG-3′, NF-κB (mouse): 5'- ACACCTCTGCATATAGCGGC-3' and 5'- CCCGGAGTTCATCTCATAGTTGT-3', TNF-α (mouse): 5′-AGGCTGCCCCGACTACGT-3′ and 5′-GACTTTCTCCTGGTATGAGATAGCAAA-3′, IL-1β(mouse):5′-CTTTCGACAGTGAGGAGAATGAC-3′ and 5′-CAAGACATAGGTAGCTGCCACAG-3′, IL-10(mouse): 5′-GGTTGCCAAGCCTTATCGGA-3′ and 5′-ACCTGCTCCACTGCCTTGCT-3′, GAPDH (mouse): F-5′-TGAAGCAGGCATCTGAGGG-3′ and R-5′-CGAAGGTGGAAGAGTGGGAG-3′. QPCR analysis was conducted on a MyiQ2 PCR thermocycler instrument (Sigma-Aldrich, Beijing, China). The expression level of GAPDH was used to standardize mRNA expression and use multiple variation=$2^{-\Delta\Delta CT}$ calculation.

Western blot

Exosome pellets or cells were lysed by using RIPA buffer (Beyotime, Beijing, China). The concentration of total proteins was detected by using a commercialized kit (Beyotime, Beijing, China). Proteins (60 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane (ISEQ00010, Sigma-Aldrich, Beijing, China). Milk powder (5%) diluted with Tris buffer brine (TBS) containing 0.05% Tween 20 was used to block the nonspecific sites (37573, TBST, Thermo Scientific, Beijing, China). Then, the following primary antibodies were added to the membranes and were incubated overnight at 4 °C: mouse polyclonal antibodies for CD63, Apo-A1, Bax, Bcl-2, TLR2, TLR4, TLR6, NF-κB and β-action (1:1000 dilution). After three times washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (A0208, 1:2000 dilution; Beyotime, Beijing, China). Blots were evaluated by enhanced chemiluminescence. β-Actin as an internal reference protein. The expression of the above protein levels was quantified densitometrically using the Quantity One software (Bio-Rad, Hercules, USA).

Statistical analysis

SPSS (IBM SPSS Statistics 19.0) was used for all statistical analyses. Data were expressed as Mean ± SD. The differences between the two groups were analyzed by *t*-test. Statistical comparisons among three or more groups were analyzed by one-way ANOVA. The difference with $p < 0.05$ was considered statistically significant.

Results

Characterization of HC11-derived exosomes

As shown in Fig 1A, the morphology analysis of isolated exosomes with or without miR-155 inhibitor by TEM demonstrated the appearance of exosomes with a size range of 100–1000 nm. Results of Nanoparticle-tracking analysis (NTA) on the exosomes with or without miR-155 inhibitor showed that most of the exosomes had an average size of around 130 nm (Fig 1B). The results of the western blot showed that the expression of CD63 and Apo-A1 was observed in HC11-derived exosomes with or without miR-155 inhibitor (Fig 1C). HC11-derived exosomes with or without miR-155 inhibitor were labeled with PKH26 (red). After 24 h, exosomes (red fluorescence) and the nuclear (blue fluorescence) of the HC11 cells were found in the cytoplasm, which indicated that exosomes could be taken up by the HC11 cells (Fig 1D).

Quantify the efficiency of exosome-mediated miRNA delivery and the expression of miR-155 in HC11 cells transfected with exosomes with or without miR-155 inhibitor

As shown in Figure 2A, q-PCR results showed that the expression level of miR-155 was significantly reduced in exosomes transfected with miR-155 inhibitor. In addition, the expression level of miR-155 was significantly decreased in HC11 cells treated with exosomes transfected with miR-155 inhibitor (Figure 2B).

The effects of miR-155 inhibitor-loaded exosomes on the expression of miR-155 and apoptosis in LPS-stimulated HC11 cells

The expression level of miR-155 was up-regulated significantly in LPS-induced HC11 cells ($P<0.001$) and LPS-induced HC11 cells transfected with blank exosomes, however, it was decreased obviously after transfected with exosomes containing miR-155 inhibitor ($P<0.001$, comparing with LPS+Exo group) (Fig 3A). The results of TUNEL staining showed that cell apoptosis was promoted in LPS-induced HC11 cells ($P<0.001$) and LPS-induced HC11 cells transfected with blank exosomes, however, it was inhibited obviously after transfected with exosomes containing miR-155 inhibitor ($P<0.001$, comparing with

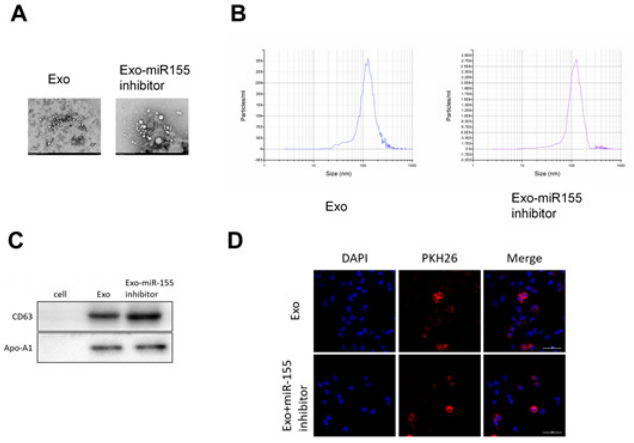

Figure 1. Characterization of exosomes. (A) Exosomes were identified by TEM, (B) NanoSight and (C) Western blot, and (D) Fluorescence microscope images showed the labeled exosomes in the cytoplasm of HC11 cells.

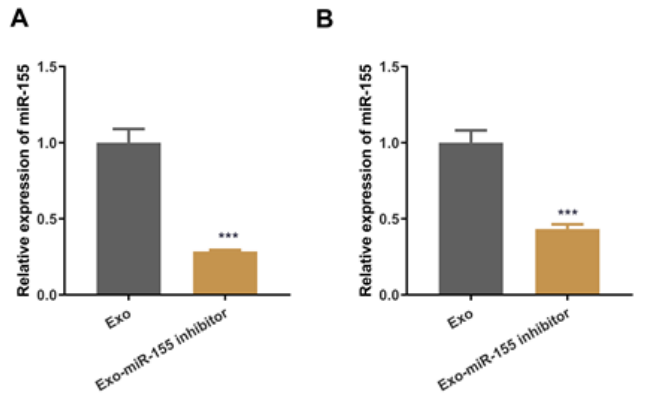

Figure 2. Quantify the efficiency of exosome-mediated miRNA delivery and the expression level of miR-155 in HC11 cells transfected with exosomes with or without miR-155 inhibitor. (A) The expression level of miR-155 in exosomes was detected using q-PCR. (B) The expression level of miR-155 in HC11 cells transfected with exosomes with or without miR-155 inhibitor was detected using q-PCR. ***p<0.001 (*vs* Exo group).

LPS+Exo group) (Fig 3B). The level of Bax was increased significantly in LPS-induced HC11 cells (P<0.001) and LPS-induced HC11 cells transfected with blank exosomes, however, it was decreased obviously after transfected with exosomes containing miR-155 inhibitor (P<0.001, comparing with LPS+Exo group). The changing trend of Bcl-2 expression was opposite to that of Bax. The alterations in expression levels of Bax and Bcl-2 further validated the apoptosis in HC11 cells (Fig 3C).

The effects of miR-155 inhibitor-loaded exosomes on the expression of genes related to TLRs/NF-κB signaling pathway and the levels of inflammatory cytokines in LPS-stimulated HC11 cells

The expression levels of TLR2, TLR4, TLR6, and NF-κB were increased significantly in LPS-induced HC11 cells (P<0.001) and LPS-induced HC11 cells transfected with blank exosomes, however, it was decreased obviously after transfected with exosomes containing miR-155 inhibitor (P<0.001, comparing with LPS+Exo group) (Fig 4A, 4B). The expression levels of TNF-α and IL-1β on mRNA level were increased significantly in LPS-induced HC11 cells (P<0.001) and LPS-induced HC11 cells transfected with blank exosomes, however, it was decreased obviously after transfected with exosomes containing miR-155 inhibitor (P<0.001, comparing with LPS+Exo group). However, the change of IL-10 level is opposite to the above genes. The changing trend in the concentration of TNF-α, IL-1β, and IL-10 was similar to the expression of their mRNA (Fig 4C, 4D).

Discussion

Mastitis is a kind of common disease and is mainly induced by pathogens, while Staphylococcus aureus is an important pathogen causing mastitis in women (19). Exosomes are involved in the regulation of immune responses in many diseases. Exosomes, used as emerging carriers of small RNAs and proteins, have attracted extensive attention in the field of drug delivery (20). Lots of studies have shown that Engineered exosomes containing miRNA could be new means to treat diseases including autoimmune diseases, inflammation, and cancer (21-23). In the mammary gland, most miRNAs are involved in immune-related functions and undergo expression pattern changes in the process of lactation (24). miRNAs in cells as well as in exosomes were involved in immune regulation and alleviating mastitis (25, 26). CD63 and Apo-A1 are widely recognized exosome markers that contribute to identifying exosomes secreted by cells (27, 28). In our study, the levels of CD63 and Apo-A1 were increased in HC11 cells transfected with exosomes with or without miR-155 inhibitor, which indicated that the synthesis of exosomes was successful. As PKH lipophilic dyes, PKH26 has been widely used to track the exosomes in cells (29). In this study, we found the exosomes (red) and the nuclear (blue) of the HC11 cells were found in the cytoplasm, which indicated that exosomes could be taken up by the HC11 cells. The analysis of the efficiency of exosome-mediated miRNA delivery and the expression level of miR-155 in HC11 cells transfected with exosomes with or without miR-155 inhibitor also indicated that exosomes containing miR-155 inhibitor could significantly inhibit the expression of miR-155 and exosomes containing miR-155 inhibitor could be applied in the following studies.

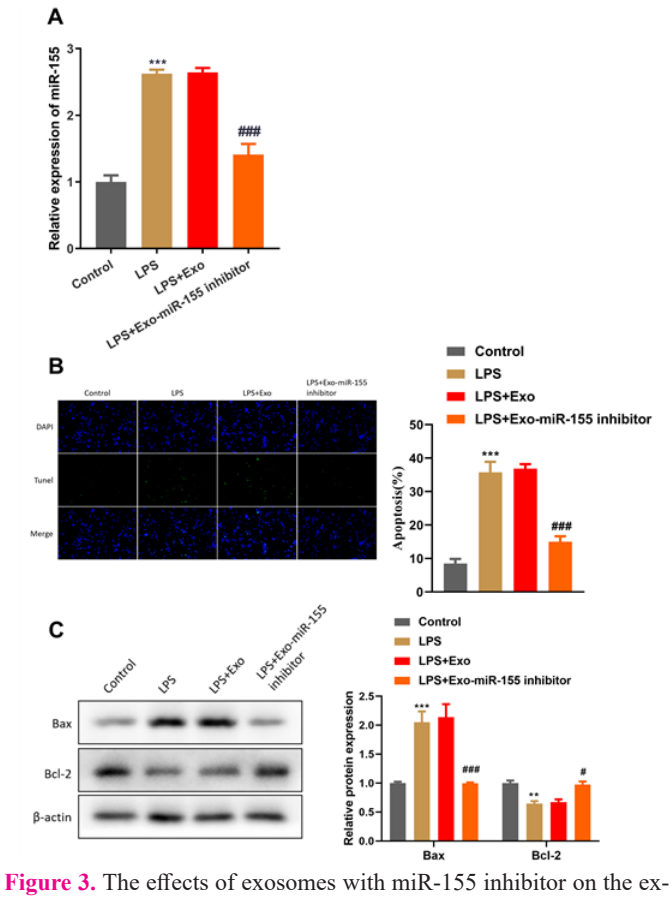

Figure 3. The effects of exosomes with miR-155 inhibitor on the expression of miR-155 and apoptosis in LPS-stimulated HC11 cells. (A) The expression level of miR-155 in LPS-induced HC11 cells transfected with exosomes with or without miR-155 inhibitor was detected using q-PCR. (B) TUNEL staining in LPS-stimulated HC11 cells transfected with exosomes with or without miR-155 inhibitor. The blue color indicated the nucleus and the green color indicated TUNEL positive. Scale bar = 30 μm. Data are shown as mean±SD. (C) The expression of Bax and Bcl-2 on protein level in LPS-induced HC11 cells transfected with exosomes with or without miR-155 inhibitor were detected using WB. **p<0.01, ***p<0.001 (vs Exo group); #p< 0.05, ###p< 0.001 (*vs* LPS+Exo group).

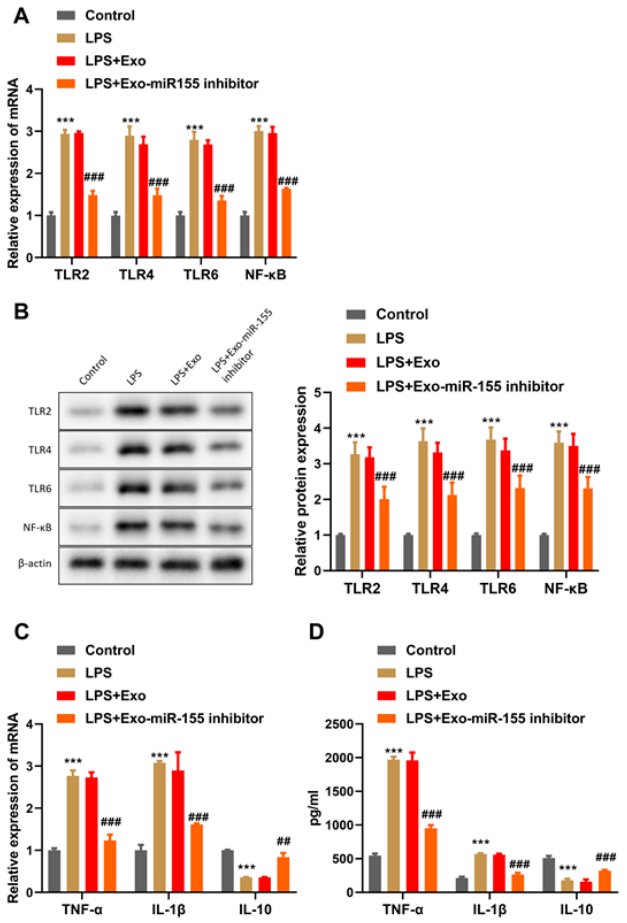

Figure 4. The effects of exosomes with miR-155 inhibitor on the expression of TLRs/NF-κB signaling pathway-related genes and the levels of inflammatory cytokines in LPS-stimulated HC11 cells. (A) The expression level of TLR2, TLR4, TLR6, and NF-κB in LPS-induced HC11 cells transfected with exosomes with or without miR-155 inhibitor was detected using q-PCR. (B) The expression of TLR2, TLR4, TLR6, and NF-κB on protein level in LPS-induced HC11 cells transfected with exosomes with or without miR-155 inhibitor were detected using WB. (C) The expression level of TNF-α, IL-1β, and IL-10 in LPS-induced HC11 cells transfected with exosomes with or without miR-155 inhibitor was detected using q-PCR. (D) The concentration of TNF-α, IL-1β, and IL-10 in LPS-induced HC11 cells transfected with exosomes with or without miR-155 inhibitor were detected using ELISA. ***p<0.001 (*vs* Exo group); ##p< 0.01, ###p< 0.001 (*vs* LPS+Exo group)..

As a commonly known endotoxin, lipopolysaccharide (LPS) is a widely occurring and widely studied pathogen-regarded protein (30). The immune system senses extracellular LPS via toll-like receptor (TLR) -4 (31). Previous studies have shown that a mastitis model induced by LPS could be obtained by injecting exogenous LPS into the mammary gland (32). In this study, the expression level of miR-155 was increased in LPS-induced HC11 cells, while it was decreased obviously after treatment with exosomes containing miR-155 inhibitor, which indicated that miR-155 might be up-regulated in mastitis. To further reveal the underlying mechanism regarding this phenomenon, we analyzed the apoptosis in LPS-induced HC11 cells transfected with exosomes with or without miR-155 inhibitor by TUNEL and the expression of Bax and Bcl-2 by q-PCR. Results indicated that LPS could promote apoptosis in HC11 cells, which was consistent with previous research results (33). However, the promoting effect was significantly recovered by miR-155 inhibitor-loaded exosomes, which indicated that miR-155 might alleviate mastitis by inhibiting apoptosis. TLR2, TLR4, and TLR6 are key genes for TLRs signaling and are involved in the progress of mastitis (34). NF-κB and TLRs played key roles in the treatment of LPS-induced mastitis with certain drugs (35). In our study, we found TLR2, TLR4, TLR6, and NF-κB were up-regulated in LPS-induced HC11 cells, which was consistent with previous research results (32, 36). However, the alterations in the expression of the above genes were significantly recovered by adding miR-155 inhibitor-loaded exosomes, which indicated that miR-155 might alleviate mastitis via the TLRs/NF-κB signaling pathway. The previous study has shown that the levels of TNF-α, IL-1β, and IL-10 were increased in Mastitis induced by LPS (37). We also found the same changes in the levels of TNF-α, IL-1β, and IL-10 in LPS-induced HC11 cells in our study. After the treatment of exosomes with miR-155 inhibitor, the levels of TNF-α, IL-1β, and IL-10 in LPS-induce HC11 cells were obviously decreased, which indicated that miR-155 might improve mastitis by alleviating inflammatory responses.

In summary, we for the first time found that engineered exosomes containing miR-155 could alleviate mastitis via down-regulation of TLRs/NF-κB signaling pathway to inhibit inflammatory responses and miR-155 might be a potential therapeutic target in mastitis.

Interest conflict

The authors state that there are no conflicts of interest.

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article.

Authors' contribution

Jinye Lu and Beibei Gu had an equal role in study design, statistical analysis, and manuscript writing; Xinlu Han and Yinnan Feng had an equal role in data collection and analysis.

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