

Intranasal delivery of mesenchymal stem cell exosomes modulates the immune response of allergic rhinitis via the MAPK pathway in a mouse model

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Abstract

Background Allergic rhinitis (AR) is a non-infectious chronic inflammatory disease of the nasal mucosa, which is mainly mediated by IgE after the body is exposed to allergens. It has been shown that transplantation of human mesenchymal stem cells (MSCs) into AR animal models can improve the AR behavioral phenotype. Furthermore, there are recent studies that states exosomes are the main mediators of MSC therapy. However, the effect of exosomes on AR has not been investigated. **Methods** In the established AR mouse model, different concentrations of MSC-derived exosomes (MSCs-Exo) were applied via intranasal delivery. The AR symptom scores, the eosinophils in the nasal mucosal section, the inflammation infiltration in the spleen section, and IgE, ovalbumin-specific IgE (OVA-sIgE), histamine, IgG1, IgG2a and other Th1/Th2 related inflammatory factors were evaluated by Hematoxylin–eosin staining, Elisa, real-time PCR. **Results** We found that intranasal administration of MSCs-Exo could not only reduce the behavior of nasal scratching and sneezing in mice, but also cause a decline in related immune indicators via the MAPK pathway, including the spleen index, tissue staining, and the expression of inflammation-related cytokines. Moreover, we found that the optimal concentration of MSCs-Exo was 4×10^8 /mL. **Conclusion** The significant beneficial effects of exosomes may be exploited to develop a new, non-invasive treatment strategy for AR.

INTRODUCTION

Allergic rhinitis (AR) is a non-infectious inflammatory disease of the nasal mucosa which is mainly mediated by IgE and driven by Th2 cells after exposure to allergens. The main clinical symptoms are sneezing, a runny nose, nasal itching, and nasal congestion¹. It has been reported that 10–40% of adults worldwide suffer from AR, and in developing countries, the situation is even worse, as the number of cases of AR rapidly increased in recent decades, especially in China². The main treatment methods for AR are allergen avoidance, drug therapy, and specific immunotherapy³. However, drug therapy can only manage symptoms and cannot reverse or alleviate the immune imbalance. Although specific immunotherapy can desensitize patients to allergens and prevent the development of lesions, it has a long treatment cycle, which tends to result in poor patient compliance and unproductive treatments⁴. Therefore, it is urgent to find new directions for effective treatment.

Mesenchymal stem cells (MSCs) have the potential for self-renewal and multi-directional differentiation⁵ and have immunomodulatory functions⁶. Previous studies have shown that transplantation of MSCs via the tail vein into an AR mouse model can improve symptoms^{7, 8}. Xenogeneic MSCs have also been proven to exert similar therapeutic effects in AR mice⁹. In addition, MSCs can also reduce the immunological indicators and adjust the Th1/Th2 reaction balance in AR patients¹⁰. The application of MSCs has resulted in definite curative effects. However, MSCs have several disadvantages, including vascular obstruction caused

by the large cells¹¹, unpredictable differentiation in the host¹², and reported concerns regarding the cancer progression^{13, 14}.

It has also been proven that exosomes secreted by MSCs (MSCs-Exo) have the same efficacy as MSCs; more importantly, transplantation with its associated risks can be avoided¹⁵. Exosomes are homogenous vesicles with a diameter of 30–150 nm secreted by a variety of cells¹⁶. The vesicles contain different types of proteins, nucleotides, and other substances. Cells can swallow the vesicles through endocytosis so that the active substances in the vesicles can function inside the cells¹⁷. MSCs-Exo can mimic the functions of MSCs and exert similar immunomodulatory effects¹⁸. Moreover, exosomes do not have a nuclear structure and cannot be amplified in the host, and thus they are safer in clinical application¹⁹. However, the clinical use of MSCs-Exo to treat AR has never been investigated. In this paper, we show that AR mice treated with MSCs-Exo by intranasal administration showed a significant improvement in behavior and histology via MAPK signaling.

METHODS AND MATERIALS

Animals

Female BLAB/c mice were purchased from Nanjing Medical University and were bred in Nanjing Drum Tower Hospital. All mice were nurtured in ventilated cages and had free access to water and food. The protocols were approved by the Animal Care and Use Committee of Nanjing Medical University and animals were treated following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All operations were carried out in accordance with the approved procedures.

AR model and MSCs-Exo treatment

BALB/c mice were sensitized with ovalbumin (OVA) (Sigma-Aldrich, A5503) and aluminum hydroxide gel (Thermo Scientific, 77161) to establish the AR model. At 4 weeks of age, the mice were randomly divided into the control group, the AR group, and the MSCs-Exo treatment group. AR mice were given an intraperitoneal injection of 100 μ g OVA in 100 μ l PBS combined with 100 μ l aluminum hydroxide gel on days 1, 5, and 10 and were treated intranasally with 20 μ l 10% OVA in PBS per nostril every day from day 15 to 21. Mice in the control group were given an intraperitoneal injection of 100 μ l PBS combined with 100 μ l aluminum hydroxide gel, and they were treated intranasally with 20 μ l PBS per nostril every day. Mice in the MSCs-Exo treatment group were given an intraperitoneal injection of 20 μ g of OVA in 100 μ l PBS combined with 100 μ l aluminum hydroxide gel on days 1, 5, and 10; were treated intranasally with 20 μ l 10% OVA in PBS per nostril every day from day 15 to 21; and were treated intranasally with 20 μ l MSCs-Exo at different concentrations per nostril every day. Administration was performed using a 10- μ L pipette with no anesthetics.

Behavioral tests

After the last intranasal administration of 10% OVA on day 21, mice were separately placed in a 10 \times 10 \times 15 cm³ cage. After waiting for 10 min, their symptoms were observed for 10 min. The behaviors that were observed and quantified included nose scratching and sneezing.

Determination of spleen index

The spleen index was calculated according to the following formula: spleen index (mg/g) = spleen weight (mg) \div animal body weight (g).

Nasal lavage fluids and serum collection

At 24 h after the last intranasal administration, mice were sacrificed by cervical dislocation. PBS (1 ml) was injected into the posterior nostril and a 1.5-mL EP tube was used to collect the nasal lavage fluid at the anterior nostril. The nasal lavage fluid was centrifuged at 2000 rpm for 7 min at 4°C and stored at -20degC until analysis. Blood samples were collected from the orbital venous plexus by capillaries. The blood samples were rested for 60 min, centrifuged at 4000 rpm for 10 min at 4degC, and stored at -20degC until analysis.

Isolation of exosomes

The human umbilical cord was taken from a full-term infant, cut into small pieces, and cultured. Cells were split when the cells reached 80–90% confluence. In the following procedures, only P5 generation cells were used. After the cells grew to 70% confluence, the culture medium was discarded, the cells were washed thoroughly with PBS, and the medium was replaced with serum-free medium for 48 h. Next, the conditioned medium was collected, centrifuged at 1000 $\times g$ for 20 min to remove cell debris, and centrifuged at 2000 $\times g$ for 20 min, and the supernatant was filtered using a 220-nm filter. Next, the filtered supernatant was centrifuged at 10,000 $\times g$ for 60 min at 4degC, and the supernatant was filtered using a 220-nm filter again. The filtered supernatant was centrifuged at 100,000 $\times g$ for 3 h at 4degC, the supernatant was discarded, the pelleted exosomes were diluted in 1 ml of pulse medium, and the effective diameter of exosomes was assessed by dynamic light scattering (DLS, Brookhaven Instruments).

Transmission electron microscopy

For transmission electron microscopy (TEM), exosomes were isolated as described and directly fixed in 200 μ l of 2% paraformaldehyde. Exosome preparations (20 μ l) were allowed to adsorb in a 75 mesh Formvar/carbon coated grid for 30 min at room temperature. Grids were then washed with PBS (membrane side facing down) and dried using a filter paper. TEM was performed using a Hitachi H-9000 transmission electron microscope at 300 kV, and images were captured using a slow-scan CCD camera.

RNA extraction and real-time PCR

The spleen was taken from the sacrificed mice for RNA extraction using TRIzol Reagent (ThermoFisher, 15596026). RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1622). The concentration and integrity of RNA and cDNA were measured using a NanoDrop (Thermo Fisher, 2000). The mRNA expression values of the genes were normalized to the mRNA expression of *actin*. The results were calculated using the comparative cycle threshold ($\Delta\Delta Ct$) method. The primers used were as follows: IL-4(F): GGTCTCAACCCCGAGCTAGT; IL-4(R): GCCGATGATCTCTCTCAAGTGAT; IL-5(F): CTCTGTTGACAAGCAATGAGACG; IL-5(R): TCTTCAGTATGTCTAGCCCTG; IL-6(F): TAGTCTTCTACCCCAATTTCC; IL-6(R): TTGGTCCTTAGCCACTCCTTC; IL-10(F): GCTCTTACTGACTGGCATGAG; IL-10(R): CGCAGCTCTAGGAGCATGTG; IL-13(F): CCTGGCTCTTGCTTGCTT; IL-13(R): GGTCTTGTGTGATGTTGCTCA; IL-17(F): TTTAACTCCCTTGCGCAAAA; IL-17(R): CTTTCCCTCCGCATTGACAC; ICAM-1(F): GTGATGCTCAGGTATCCATCCA; ICAM-1(R): CACAGTTCTCAAAGCACAGCG; IFN- γ (F): ATGAACGCTACACACTGCATC; IFN- γ (R): CCATCCTTTTGCCAGTTCCTC; CCL-11(F): GAATCACCAACAACAGATGCAC; CCL-11(R): ATCCTGGACCCACTTCTTCTT; TGF- β 1(F): TCTGCATTGCACTTATGCTGA; TGF- β 1(R): AAAGGGCGATCTAGTGATGGA; TGF- β 2(F): CCGCTGCATATCGTCCTGTG; TGF- β 2(R): AGTGGATGGATGGTCCATTACA; IKK(F): ACAGCCAGGAGATGGTACG; IKK(R): CAGGGTGACTGAGTCGAGAC; IKK(F): GCGGATGCCGATGAATGGT; IKK(R): TGACGTAGCCAAAGACTAAGGG; JUN(F): CCTTCTACGACGATGCCCTC; JUN(R): GGTCAAGGTCATGCTCTGTTT; JNK(F): CAGACTGTACCCTCAAGATCCT; JNK(R): ATGCACCCGACAGACCAGA; ERK(F): GGTGTTCCCAAATGCTGACT; ERK(R): CAACTTCAATCCTCTTGTGAGGG; FOS(F): CGGGTTTCAACGCCGACTA; FOS(R): TTGGCACTAGAGACGGACAGA.

ELISA

Standard wells and blank wells were set in accordance with the protocol of the Bioswamp ELISA kit. Serum (40 μ l) was added to each sample well, and 10 μ l biotinylated antibody and 50 μ l HRP-conjugated reagent were added to each well except the blank well. After incubating for 30 min at 37°C, washing buffer was added to every well and samples were rested for 30 s five times. Then, chromogen solution was added to each well and samples were incubated for 10 min at 37°C. Lastly, stop solution was added to terminate the reaction and the optical density (OD) at 450 nm was measured within 15 min.

Hematoxylin–eosin staining

After mice were sacrificed, their entire heads and spleens were soaked in 10% neutral formalin fixative for 24 h, because the nasal cavity of BALB/c mice is small. Afterwards, the heads were soaked in 0.5 M ethylene diamine tetraacetic acid for decalcification for 4 weeks. After the mouse skull was softened, the tissues were conventionally dehydrated, embedded in paraffin, and sectioned in the coronal position (about 3 μm thick slices). The histopathological changes of the nasal mucosa were visualized with hematoxylin–eosin staining. The same steps were applied to the spleens.

Statistical analysis

At least three independent experiments were performed for each experimental condition. Data were analyzed by GraphPad Prism 5 software and are shown as mean \pm SEM. In addition, two-tailed, unpaired Student *t*-tests were used. Animals were randomly assigned during collection, but the data analysis was single-masked.

RESULTS

Characterization of MSCs-Exo

MSCs-Exo have been recognized as a promising substitution for MSCs. Our size distribution analysis revealed that the MSCs-Exo were around 100 nm in diameter (Figure 1B). TEM analysis of the morphology further confirmed the exosomes' identity (Figure 1A).

Intranasal delivery of MSCs-Exo relieves AR symptoms

We used an AR mouse model to investigate the role of MSCs-Exo in AR (Figure 2A). We measured the number of sneezing and nose rubbing motions during 10 min after the last intranasal administration of OVA. The results showed that the mice in the AR group sneezed and rubbed more frequently than those in the control group. Moreover, the numbers of sneezing and rubbing events were significantly lower in the MSCs-Exo treatment group compared to the AR group (Figure 2B). In conclusion, intranasal treatment with MSCs-Exo could notably reduce sneezing and rubbing frequencies, thus indicating that MSCs-Exo could relieve the symptoms in AR mice.

Intranasal delivery of MSCs-Exo reduces AR inflammation

To further understand the effects of MSCs-Exo on AR, the levels of different inflammation-related indicators were measured. As shown in Figure 2C, sections of the control group revealed normal nasal cavity mucosa. In the AR group, we observed more infiltrating eosinophils and lymphocytes, glandular secretion, and mucosal swelling (Figure 2D). After MSCs-Exo treatment, there were significantly fewer infiltrating cells and less glandular secretion and mucosal swelling in the nasal mucosa (Figure 2E). Additionally, the quantification of eosinophils (Figure 2F) suggested that MSCs-Exo have a therapeutic effect on AR mice. The spleen index, which also reflects the inflammatory status, demonstrated similar differences between the three groups (Figure 3A). Spleen sections from different groups are shown in Figure 3B–D. We observed apparent splenic sinus edema, splenic capsule incassation, and vasodilation in the AR group (Figure 3C), and MSCs-Exo administration reduced inflammation.

The cytokine levels in the spleen, including Th2 cytokines (IL-4, IL-5, IL-6, IL-10, IL-13, and TGF- β), a Th1 cytokine (IFN- γ), a Th17 cytokine (IL-17), an eosinophil chemoattractant (CCL-11)²⁰, and an adhesion receptor (ICAM-1)²¹, were analyzed by RT-PCR at 24 h after the last treatment. As shown in Figure 4A, IL-4, IL-5, IL-6, IL-13, CCL-11, and ICAM-1 levels were significantly decreased in the MSCs-Exo group compared to the AR group. While there were no differences in IL-17 and TGF- β levels, IL-10 and IFN- γ levels were obviously enhanced in the MSCs-Exo group compared to the AR group. The serum concentrations of histamine, OVA-IgE, IgE, IgG1, and IgG2a, and the concentrations of histamine, OVA-IgE, and IgG1 in bronchoalveolar lavage fluid (BALF), were measured by ELISA. The MSCs-Exo mice expressed low levels of OVA-IgE, IgE, and IgG1, but not IgG2a (Figure 4B and C). These results verify our hypothesis that intranasal delivery of MSCs-Exo could inhibit allergic airway inflammation and regulate the balance of Th1/Th2 in OVA-sensitized mice.

Optimal concentration of MSCs-Exo

After clarifying the therapeutic effect of MSCs-Exo, we used different concentrations of MSCs-Exo to find the optimal concentration for AR treatment. We used 4×10^6 /mL, 4×10^7 /mL, 4×10^8 /mL, 4×10^9 /mL, and 4×10^{10} /mL exosomes for intranasal administration in AR mice (Figure 5A). We found that the effect was dose-dependent (Figure 5B–E). At a concentration of 4×10^6 /mL, we observed basically no effect on the sneezing and rubbing events and inflammation-related indicators. At concentrations higher than 4×10^8 /mL, no significant further improvement in the inflammatory symptoms of the mice was observed. This result suggests that the optimal concentration of MSCs-Exo to reduce allergic inflammation in mice is around 4×10^8 /mL.

MSCs-EXO suppress inflammation via the MAPK pathway

As shown in Figure 6, the mRNA levels of *JUN*, *FOS*, *ERK*, *JNK*, *IKB*, and *IKK* were significantly higher in the AR group than in the control group, while the mRNA levels of *JUN*, *FOS*, *ERK*, and *JNK* were significantly lower in the MSCs-Exo group than in the AR group but higher than in the control group. Furthermore, there were no differences in the mRNA levels of *IKK* and *IKB* between the AR group and the MSCs-Exo group. These results imply that AR might stimulate inflammation by activating the MAPK and NF- κ B pathways, while MSCs-Exo alleviates inflammation by inhibiting the MAPK pathway.

DISCUSSION

We have verified that the AR mouse model was successfully established. We first showed that intranasal delivery of MSCs-Exo could not only relieve the main AR symptoms, sneezing and runny nose, but also decrease various inflammation indicators, including the spleen index, tissue staining, and the expression of inflammation-related cytokines. Moreover, we found that the optimal concentration of MSCs-Exo was 4×10^8 /mL. Besides, we demonstrated that the MAPK and NF- κ B pathways play an important role in inflammation, and that MSCs-Exo ameliorated AR by inhibiting the expression of MAPK pathway proteins, which modulated the immune response in the AR mouse model.

MSCs exert immunoregulatory effects by inhibiting T cell proliferation, inactivating allogeneic T cells, influencing T cell apoptosis²², and inhibiting B cell proliferation to decrease antibody secretion²³. Furthermore, MSCs inhibit the differentiation and maturation of dendritic cells, resulting in a decline in the ability to activate T cells²⁴. MSCs exert the above immunomodulatory effects mainly through the paracrine secretion of exosomes²⁵. The presence of microvilli and columnar cells in the nasal cavity can intensify drug absorption²⁶, and intranasal administration is not only safe but also convenient to operate. These advantages increase its feasibility for clinical application. Therefore, we chose to study the therapeutic effect of MSCs-Exo on AR by intranasal delivery; our results verify that intranasal administration of MSCs-Exo works not only locally but also systemically.

After exposure to the specific allergen, IgE mediates mast cell degranulation, causing itching and sneezing and further promoting hemangiectasis and increasing secretion of submucosal glands, thus resulting in obstruction and rhinorrhea. The immunohistological characteristics of AR include the infiltration of eosinophils and the predominant expression of cytokines secreted by Th2 cells, with a reduced activity level of Th1 cells, which contributes to the late-phase response such as nasal obstruction and hyperreactivity^{27, 28}. Our results correspond with the mechanism underlying AR described above. In the AR model, IgE, OVA-IgE, IgG1, and histamine levels in BALF and serum and Th2 cytokine levels increased, corresponding to severe local and systematic symptoms. After intranasal administration of MSCs-Exo, the decrease in IgE, OVA-IgE, and histamine levels relieved the classic symptoms; the decrease in CCL-11 levels is linked to lower eosinophil infiltration; the change in ICAM-1 and Th2 cytokine levels is linked to reduced inflammation; and increased IFN- γ levels can stimulate the expression of IgG2a and inhibit the production of IgG3, IgG1, IgG2b, and IgE²⁹, thus further exerting a therapeutic effect on AR. Many studies have also demonstrated that after intravenous treatment with different kinds of exosomes in an asthma mouse model, inflammation of lung tissues decreased, and Th2 cytokine and inflammatory cytokine levels were lower in serum and BALF³⁰⁻³². All these results imply that intranasal delivery of MSCs-Exo has an effect similar to that of intravenous

delivery to suppress allergic reactions.

In order to pave the way for clinical application in the near future, we examined the effects of different doses, and found that the optimal concentration is 4×10^8 /mL. When the concentration of MSCs-Exo is lower than 4×10^8 /mL, the efficacy decreases significantly. Once the concentration is higher than 4×10^8 /mL, the curative effects do not further increase significantly. The data show that MSCs-Exo works in a dose-dependent manner, which supports future clinical research.

The activation of NF- κ B plays an important role in allergic diseases, inducing the accumulation of inflammatory cells³³. When activated by inflammatory signals, the released NF- κ B induces the transcription of many inflammatory cytokines, which are related to the pathogenesis of asthma³⁴. MAPKs are involved in many different cellular events, including allergic diseases³⁵. Mammalian MAPKs are mainly divided into three categories: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38³⁶. The activation of MAPK is critical for the production of inflammatory cytokines³⁷, and functional differentiation into Th1 or Th2 subsets³⁸. Notably, inhibition of ERK promotes the transition of Th2 lymphocytes to Th1³⁹. Our results showed that the expression of genes related to the NF- κ B and MAPK pathways increased significantly in AR mice, but after MSCs-Exo treatment, the expression of genes related to the NF- κ B pathway did not decrease significantly, while MSCs-Exo treatment significantly inhibits ERK, JNK, JUN, and FOS expression. The results confirm that the NF- κ B and MAPK pathways play important roles in AR and indicate that MSCs-Exo do not reduce the activation of NF- κ B to alleviate the symptoms of AR. MSCs-Exo inhibit inflammation by restricting OVA from affecting the MAPK pathway in AR mice.

While our results have demonstrated that intranasal delivery of MSCs-Exo is an effective therapy for AR, the specific mechanism has not been adequately revealed. It has not been researched how the MAPK pathway regulates the expression of cytokines to play a therapeutic role in AR. The specific functional components of MSCs-Exo have not been identified, and it is unknown how long they remain active. Previously, the effects of intranasal and intravenous delivery of MSCs-Exo on the brain were compared, and intranasal delivery was more effective⁴⁰. For AR, no direct comparisons between intravenous and intranasal delivery have been made. Note also that we only studied intranasal administration of MSCs-Exo isolated from human umbilical cord; and while this extends the current treatment methods, it will be interesting to test exosomes secreted by other cells to examine the different effects.

Intranasal administration of MSCs-Exo has been researched in various diseases, including complete spinal cord injury⁴¹, microglia-mediated neuroinflammation⁴², and autism spectrum disorders⁴³. It is noteworthy that intranasal delivery of MSC-Exo could substantially expand pulmonary IL-10-producing interstitial macrophages to protect against allergic asthma in mice⁴⁴. However, research on intranasal delivery of MSC-Exo in AR is still scarce. Our results offer a theoretical and experimental basis for the future clinical local application of MSCs-Exo in the nasal cavity for the treatment of AR, which can effectively alleviate pain in AR patients and has significant clinical and social value.

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Conflict of interest

The authors declare no competing interests.

Author contributions

CY, YC, BW, XC, and XG conceived and designed the experiments. CY, YC, XX, XD, SL, HW, XW and ZX performed the experiments. CY, YC, XX, XD, SL, BW, XC, and XG analyzed the data. CY, YC, BW, XC, and XG wrote the paper. All authors read and approved the final manuscript.

Data availability statement

All data generated or analyzed during this study are included in this article.

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