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Loading Ovalbumin into Mesenchymal Stem Cell-Derived Exosomes as a Nanoscale Carrier with Immunomodulatory Potential for Allergen-Specific Immunotherapy

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Abstract

Background: Exosomes are nanoscale vesicles widely used as drug delivery systems. Mesenchymal stem cell (MSC)-derived exosomes have shown immunomodulatory potential. This study optimized loading OVA into the mice adipose tissue-derived MSC-isolated exosomes to prepare the OVA-MSC-exosome complex for allergen-specific immunotherapy.

Methods: MSCs were harvested from mice adipose tissue and characterized by flow cytometry and evaluating differentiation potential. The exosomes were isolated and characterized via Dynamic Light Scattering, Scanning Electron Microscopy, and flow cytometry. Different concentrations of ovalbumin were incubated with MSC-exosome in various durations to optimize a more suitable protocol. BCA and HPLC analysis were used to quantify, and DLS was applied to qualify the prepared formulation of the OVA-exosome complex.

Results: The harvested MSCs and isolated exosomes were characterized. Analysis of the OVA-exosome complex revealed that OVA in primary 500 µg/ml concentration and incubation for 6 h results in higher efficacy.

Conclusions: Loading OVA into MSC-derived exosomes was successfully optimized and could be administrated for allergen-specific immunotherapy in the animal model.

Keywords: Delivery system, Exosome, Mesenchymal stem cell (MSC), Ovalbumin.

Introduction

Exosomes are nanoscale vesicular particles in 30-200 nm diameters secreted by a wide variety of cells and exist in most biological fluids such as serum, urine, and saliva (1-3). High stability, low immunogenicity, suitable bioavailability, and long-term circulation are among the exciting characteristics of exosomes. In addition, the phospholipid bilayer enables exosomes to merge with the membrane of target cells and subsequently facilitates the cellular internalization of cargo (4, 5). The siRNA (6), miRNA (7), curcumin (8, 9), catalase (10), dopamine (11), paclitaxel (12, 13), and doxorubicin (14) are examples of drugs and therapeutic agents that delivered via exosome carriers in recent studies.

Based on the tissue or cell from which they originate, exosomes have various functions and mainly imitate the function of their cell of origin (15). Altogether, mesenchymal stem cell (MSC)-derived exosomes showed similar properties to these cells. In preclinical studies, accelerated tissue regeneration, regulated apoptosis, and cell proliferation modulated

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immune cell function, angiogenesis induction, and hemostasis maintenance described as features of MSC-derived exosomes (16).

Allergen-specific immunotherapy is a demonstrated, effective, and long-lasting approach to control and treat allergic disorders that regulate immune responses and suppress allergen reactivity. Nevertheless, it seems that allergen dosage and formulation is a crucial factor that affects the efficacy and safety of the immunotherapy process (17, 18). Different nanoparticles, e.g., chitosan, PLGA, and gold nanoparticles (GNP), were used for allergen encapsulation that effectively protects allergen against enzyme degradation and pH alteration, reduced administered dosage, and inhibited side effects (19). Ovalbumin (OVA) is derived from chicken eggs in large amounts and at low expenses and induces allergic pulmonary inflammation in animal models; hence it is the most widely used allergen to mimic allergy pathogenesis (20).

In the current study, loading OVA into the mice adipose tissue-derived MSC-isolated exosomes optimized to prepare the OVA-MSC-exosome complex for allergen-specific immunotherapy.

Materials and Methods

Isolation of mesenchymal stem cells from adipose tissue and characterization

Isolating mesenchymal stem cells from mice adipose tissue and their characterization was conducted based on previous studies with little modifications (21). Briefly, the sub-peritoneal adipose tissues from Balb/c mice were harvested sterile and transferred to cold Phosphate Buffer Saline (PBS). The tissues were minced into small sections and incubated with 0.075% Collagenase type-I (Merck, Darmstadt, Germany) for 45 minutes at 37 °C. Then, the 10% Fetal Bovine Serum (FBS)-contained Dulbecco's Modified Eagle's Medium (DMEM) media (Biosera, Kansas City, MO, USA) was added and centrifuged for 10 minutes at 500 g. The precipitated cells were isolated, resuspended in the 10% FBS, and 100 U/ml penicillin and 100 µg/ml streptomycin (Biosera, Kansas City, MO, USA)-enriched DMEM media, and transferred to culture flasks to incubated in 37 °C temperature, 95% humidity, and 5% CO2. After 48 hours, the fresh media was added, and non-adherent cells were removed. The media refreshing continued twice a week until the adherent cells reached 80% confluency. Also, the 0.05% trypsin/EDTA (Invitrogen, Waltham, MA, USA) was applied for cell passage.

To analyze the specific surface markers of MSCs, two positive markers (CD73 and CD105) that are specific for MSCs and two negative markers (CD31 and CD45) that are positive for hematopoietic stem cells and negative for MSCs were applied. In this case, from passages 2-3, the 1×10^5 cells were resuspended in a staining buffer (2% FBS-enriched PBS) and incubated for 30 minutes at 4 °C with 3 µl of specific anti-CD31, anti-CD45, anti-CD73, and anti-CD105 and also isotype control antibodies (Abcam, Cambridge, UK). Next, following a wash step using washing buffer, the cells were analyzed via FACS caliber flow cytometer (BD Bioscience, San Diego, CA, USA) and Flow Jo software (San Jose, CA, USA).

To examine the osteogenic differentiation potential of MSCs, cells from passages 2-3 were cultured in 24-well plates. The osteogenesis inducer media (Kiazist, Iran) consisting of 10 nM dexamethasone, 10 mM β-glycerol phosphate, and 50 µg/ml ascorbic acid biphosphate were added to the confluent cells. Osteogenesis induction was continued for three weeks, and the media was refreshed every three days. Finally, differentiated cells were fixed by paraformaldehyde solution and stained using Alizarin Red to detect mineralized matrix under microscopic evaluation.

Isolation and characterization of exosomes

Based on previous studies (7), the confluent passage two-to-five MSCs were applied for exosome isolation. In this case, cells were adapted to the FBS-free media for 72 hours. Then, the supernatants were harvested, centrifuged at 2500 g for 10 minutes at 4 °C, and filtered through a 0.22 µ pore size membrane. The collected solution was applied

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for exosome isolation via the exosome isolation kit (Exospin, MO, USA). Also, the Bicinchoninic acid (BCA) protein assay kit (Parstous biotechnology, Mashhad, Iran) was applied to evaluate the protein content of purified exosomes.

Scanning electron microscopy (SEM) (KYKY-EM 3200) and Dynamic Light Scattering (DLS) Zetasizer (Malverns Instruments Ltd, Malvern, UK) were used to characterize the exosomes' shape and size, respectively. For DLS analysis, the PBS-resuspended exosome solution was applied, while for SEM analysis, an air-dried exosome smear was coated with a thin layer of gold, then underwent microscopic analysis.

In addition, based on previous studies (22), CD9 and CD63 were considered specific markers to evaluate the surface markers of exosomes. In this case, latex bead-incubated exosomes were stained using anti-CD9 and anti-CD63 antibodies (Abcam, Cambridge, UK), and analysis was conducted using FACS caliber flow cytometer (BD Bioscience, San Diego, CA, USA) and Flow Jo software (San Jose, CA, USA).

Preparation of the OVA-loaded exosome

Based on previous studies, loading OVA into exosomes was conducted via incubation (10) with some modifications. In this case, three different concentrations (100, 300, and 500 µg/ml) of OVA solution (Becton Dickinson Co, Franklin Lakes, New Jersey, USA) in 200 µl volume were added to 200 µl of exosome solution (200 µg/ml) and incubated on a shaker at 22 °C for 6, 12, and 24 hours in triplicate. Then, to separate OVA-loaded exosomes and remove free OVA, the Exosome isolation kit (Exospin, MO, USA) was used.

Both direct and indirect methods were applied to evaluate the concentration of OVA loaded into exosomes. First, the BCA protein assay kit (Parstous biotechnology, Mashhad, Iran) and the following formula were used to assess the concentration of free OVA in the supernatant indirectly:

The OVA concentration in the exosome-OVA complex = primary concentration of OVA in the solution before incubation – the free OVA concentration in the supernatant.

Next, UV-HPLC (Hewlitt Packard 1100 series, Santa Clara, CA, USA) was applied to direct quantify the exact concentration of OVA in the exosome-OVA complex following exosome lysis by the radioimmunoprecipitation assay (RIPA) buffer (Kiazist, Iran). Also, the OVA-loaded exosomes were characterized using DLS.

Results

Characterization of MSCs

The flow cytometric analysis confirmed a high expression of MSC-specific markers (CD73 and CD105) and a low expression of hematopoietic markers (CD31 and CD45) (Fig. 1). Also, microscopic evaluations revealed typical fibroblast-like morphology of MSCs following plastic adherence (Fig. 2a). In addition, alizarin red stained-extracellular calcium deposits following *in vitro* differentiation demonstrated the osteogenic potential of MSCs (Fig. 2b).

Characterization of exosomes

Analysis of the size and shape of isolated exosomes using DLS (Fig. 3a) and SEM (Fig. 3b) revealed consistent results. In addition, flow cytometric analysis showed high percentages of CD9 and CD63 (Fig. 4).

Quantification of OVA-loaded exosomes

To assess the OVA loading efficacy into the MSC-derived exosomes, BCA and UV-HPLC were applied. As shown in Fig. 5, the HPLC analysis revealed that OVA incorporation in the primary 500 µg/ml OVA concentration was significantly higher than 100 and 300 µg/ml in all incubation times ($p<0.0001$). Also, in comparison between different incubation times for primary 500 µg/ml OVA concentration, while in 24 h and 12 h incubation times, the levels of OVA incorporation were higher than the 6 h; no significant differences were observed.

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In addition, the DLS analysis showed that longer incubation times (12 and 24 h) might negatively affect exosome size and morphology (Table 1). Hence, primary 500 µg/ml OVA concentration and 6 h incubation have shown higher efficacy for OVA incorporation into exosomes.

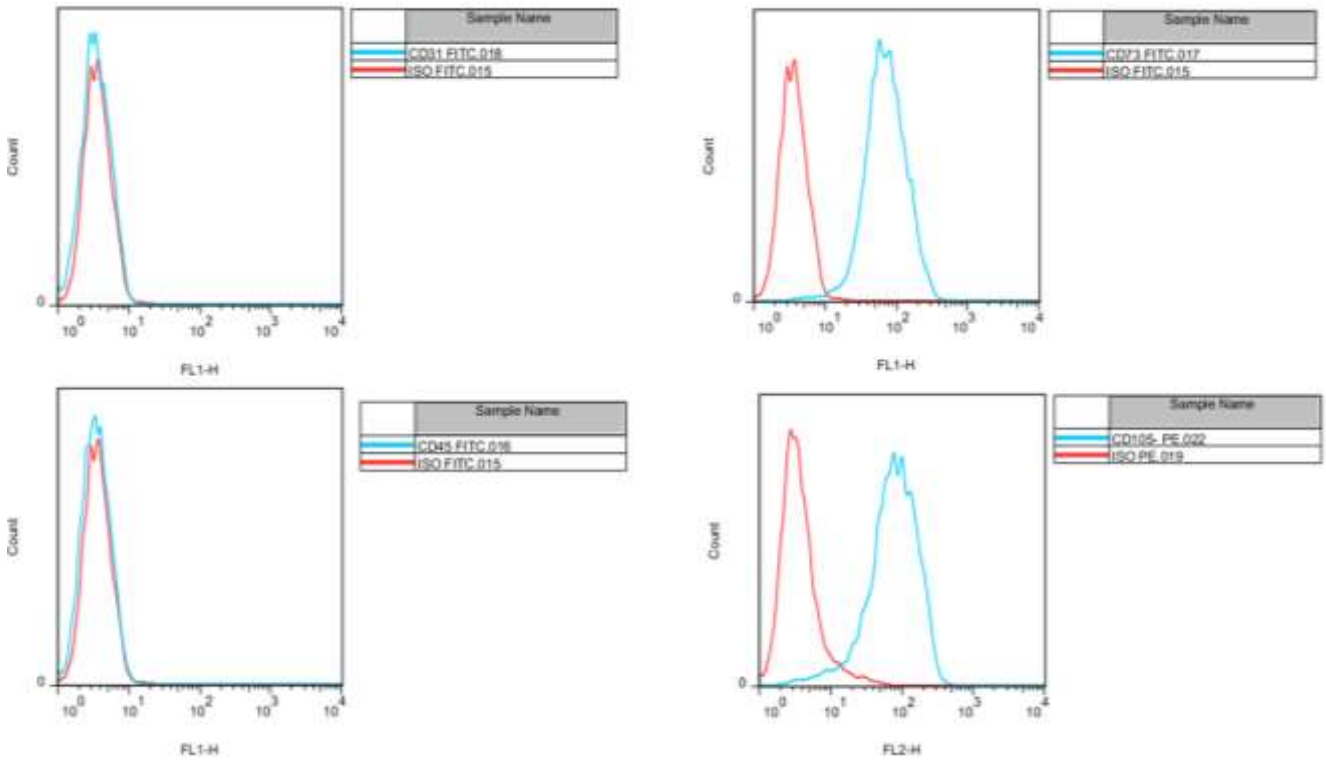

Fig. 1. Flow cytometric analysis of MSCs isolated from adipose tissue for surface expression of CD31, CD45, CD73, and, CD105 (blue graphs) in the presence of isotype control results (red graphs).

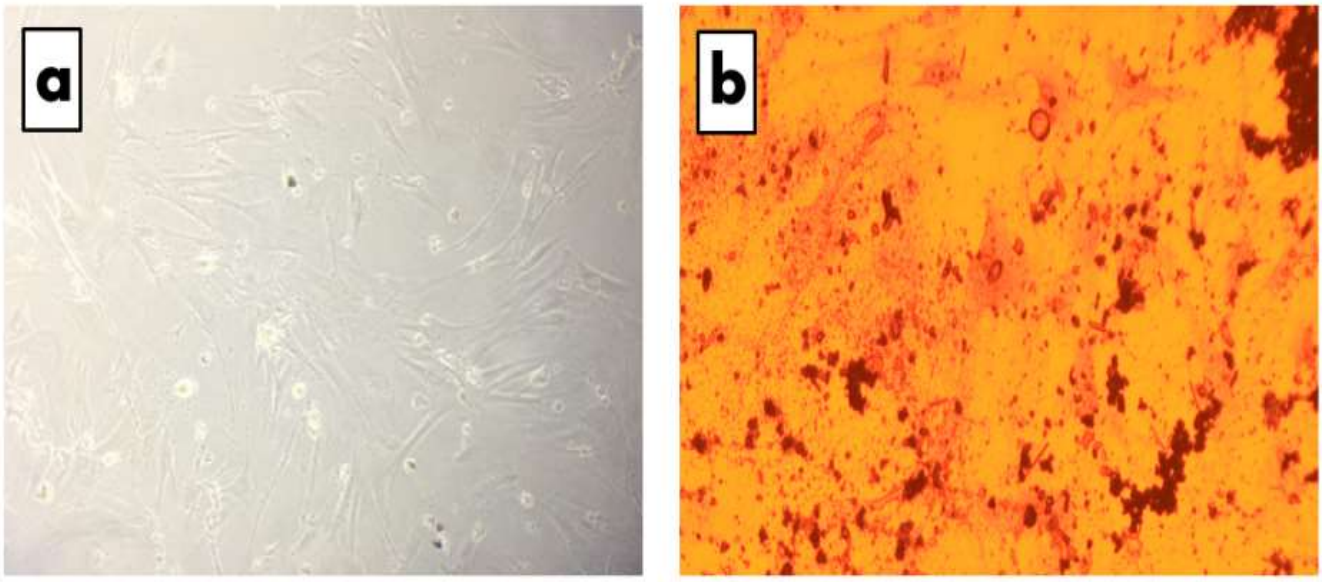

Fig. 2. Microscopic view of (a) adipose-tissue isolated MSCs and (b) osteogenic differentiated cells.

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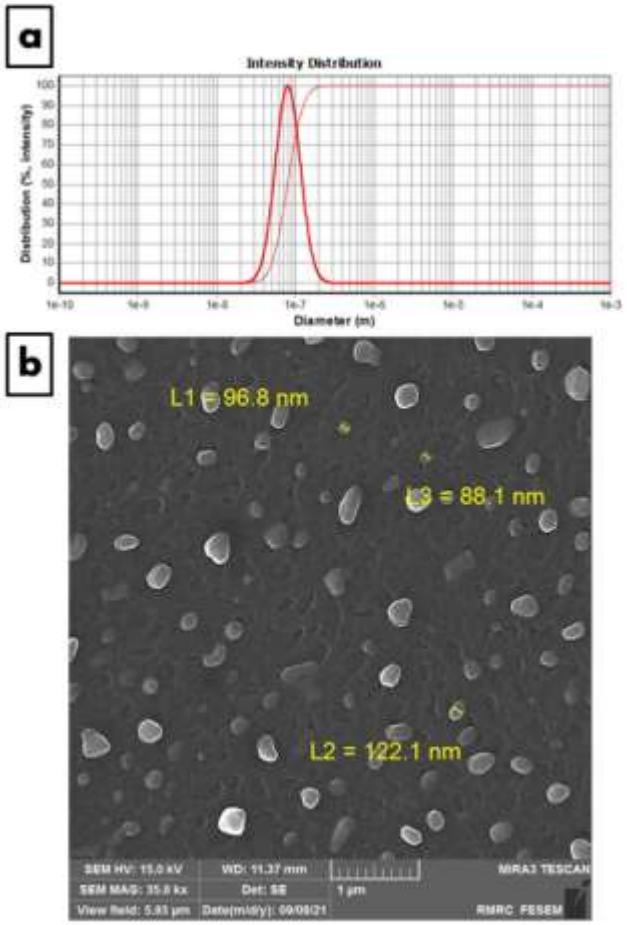

Fig. 3. The size and shape characterization of MSC-isolated exosomes via (a) DLS analysis for size; and (b) SEM analysis for shape and size.

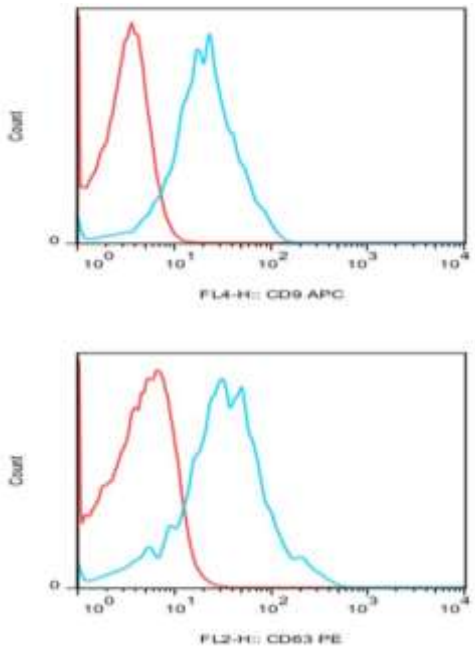

Fig. 4. Flow cytometric analysis of exosomes isolated from MSCs for surface expression of CD9 and CD63 (blue graphs) in the presence of unstained results (red graphs).

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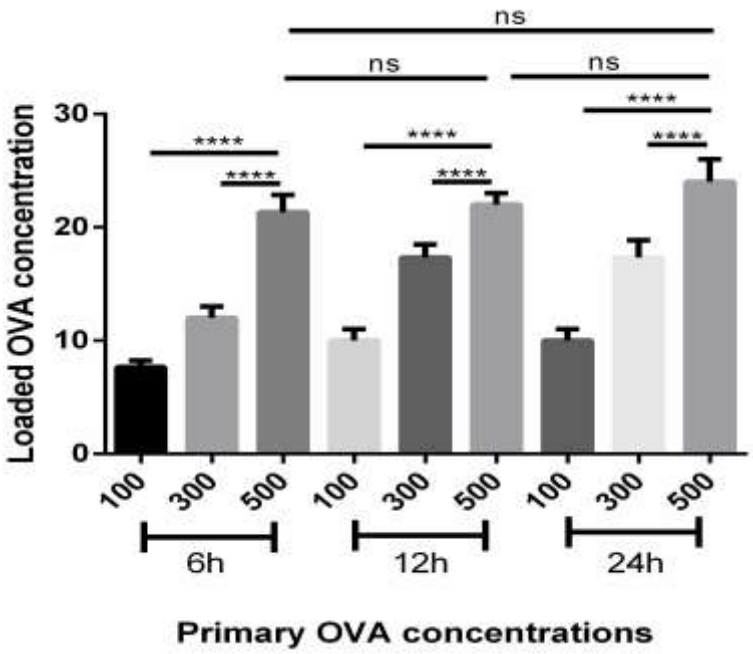

Fig. 5. The concentration of loaded OVA (µg) in different primary OVA concentrations (µg/ml) and various incubation times following HPLC analysis (in triplicate).

Table 1. Characterization of OVA-enriched exosomes in the primary 500 µg/ml OVA concentration and free exosomes following different 6, 12, and 24 h incubation times using DLS.

Incubation time (hour)	Formulation	Size (nm)	Polydispersity Index (PDI)
6	Free exosome	102.5±12.4	0.20
	OVA-enriched exosome	107.2±11.6	0.31
12	Free exosome	112.1±9.6	0.28
	OVA-enriched exosome	124.7±16.3	0.37
24	Free exosome	119.3±13.1	0.26
	OVA-enriched exosome	127.7±15.6	0.39

Discussion

In this study, following MSC and exosome isolation, we optimized the loading of the OVA into MSC-derived exosomes and prepared a nanocarrier-allergen formulation for allergen-specific immunotherapy.

MSC isolation from adipose tissue successfully proceeded, and characterizations and validations were in accordance with previous studies that harvested MSCs from mice adipose tissue (21, 23, 24). In addition, the exosome isolation and characterization were successful.

Recent studies demonstrated the delivery potential of exosomes as a natural carrier to transfer various biomolecules such as proteins, mRNA, miRNA, and siRNA (7, 10, 25). Exosome loading for drug delivery approaches was conducted in two main ways with different loading efficacy and stability. One way is loading cargo into the donor cells before

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releasing exosomes (4). Another is the direct transfer of the drug into exosomes via different routes, including incubation, electroporation, extrusion, sonication, and freeze/thaws (10). Incubation is a simple method generally conducted following incubating therapeutic agents with exosomes in the presence or absence of additional elements to facilitate diffusion based on concentration gradients (26). In the present study, loading OVA into exosomes was conducted via the incubation method at 22 ºC. In this case, successful incubation at 22 ºC was reported by Sun et al. (9), which incorporated curcumin into exosomes, and Saari et al. (12), which loaded paclitaxel into exosomes while the duration time was different. In addition, Qu et al. (11) incorporated dopamine into exosomes via incubation at room temperature (RT) in the presence of 0.02% ascorbic acid; Haney et al. (10) loaded catalase into exosomes via RT incubation in the presence or absence of saponin detergent. Hence, it seems that the incubation method is considered a reliable and straightforward method for exosome loading.

Nevertheless, Haney et al. compared the incubation method with three other approaches, including freeze/thaw cycles, sonication, and extrusion. Whether these methods showed higher loading efficacy, the analysis revealed that exosome reformation/deformation is higher compared to incubation and may affect the functions of exosomes in delivering cargo (10). It is noteworthy that incubation duration could affect the exosome morphology and further functionalities. Our study revealed that while longer durations may increase the loading efficacy, but affects the exosomes, and 6 h was optimized for incubation time.

The immunomodulatory potential of MSC-derived exosomes in ameliorating allergic airway inflammation was demonstrated in various studies (27, 28). De Castro et al. reported that adipose tissue-derived MSC-exosomes suppressed airway hyperresponsiveness and lung inflammation in the murine model of OVA-induced asthma (28), while Du et al. observed that MSC-exosomes increased IL-10 and TGF-β production by regulatory T cells and could subsequently stimulate the immunoregulatory potential of these cells (29).

Based on previous results that reported curcumin formulation in exosomes showed higher stability, solubility, and bioavailability compared to the free form (9), or loading paclitaxel into exosomes significantly increased its cytotoxicity against tumor cells (12, 13), it seems that OVA-MSC-exosome complex could potentially be administered for allergen-specific immunotherapy in the animal model as a formulation with immunomodulatory potential that originated from MSCs and reduced allergen dosage due to protective and delivery potential of exosomes which could reduce side effects and increase the efficacy of Treatment.

Ethics

The research was approved by the Institutional Animal Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.ABHC.REC.1399.011).

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Conflicts of Interest

All authors declare no potential conflict of interest.

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