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Original Article

Extracellular-vesicles derived from human Wharton-Jelly mesenchymal stromal cells ameliorated cyclosporin A-induced renal fibrosis in rats

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Abstract: Objective: To observe the therapeutic effects of human Wharton-Jelly mesenchymal stromal cells derived extracellular vesicles (MSCs-EVs) for cyclosporin-A-induced renal injury in rats and further to investigate the mechanism. Methods: EVs from MSCs were made using the ultra-centrifugation method. The cyclosporin A-induced renal injury model in rats was set up, and MSCs-EVs were administrated at d7 and d21 intravenously. The animals were sacrificed at d28, and the serum and kidneys were obtained. Renal fibrosis was assessed using Masson's staining and α-SMA IHC staining. Renal function was determined using serum creatinine. The SOD and malondialdehyde (MDA) in the renal tissues were also assayed. In vitro, HK2 cells were injured by CsA for 24 h as well as incubated with MSCs-EVs administration, and ROS and α-SMA expression were assessed. Results: In this animal study, renal fibrosis was alleviated and the epithelial-mesenchymal transition (EMT) was suppressed after the MSCs-EVs treatment compared with the control group. Renal function was also improved. The oxidative stress which was reflexed by the MDA and SOD change was declined after the EVs therapy. In vitro, NRK-52E cells were injured by CsA. The EVs therapy reduced α-SMA expression and ROS generation. Conclusion: MSCs-EVs alleviated CsA-induced renal injury and improved renal function, which could be attributed to the anti-oxidative property of MSCs-EVs.

Keywords: Mesenchymal stromal cells derived extracellular vesicles (MSCs-EVs), cyclosporin A, epithelial-mesenchymal transition (EMT), oxidative stress

Introduction

With the continuous progress of kidney transplantation, more and more patients with end-stage renal disease undergo renal transplantation and achieve good clinical results [1]. Because of the possibility of acute and chronic rejection after renal transplantation, the vast majority of patients after renal transplantation require long-term, anti-immune rejection drugs. Cyclosporin A (CsA) is one of the most commonly used immunosuppressive drugs. Studies have shown that [2], with the application of CsA, the kidney transplant survival rate and one-year survival rate of patients with a transplanted kidney were 97.1% and 89.5%, respectively. However, due to the presence of clear renal toxicity in CsA, which limits the widespread use of these drugs in the clinic, CsA-induced renal injury is mainly characterized by banded renal interstitial fibrosis, tubule atrophy, inflammatory cell infiltration, and so on. Its damage mechanism is not entirely clear [3]. It is important to note that there is no effective prevention and treatment for CsA-induced renal injury.

Our previous studies have shown that mesenchymal stem cell-derived extracellular vesicles (MSCs-EVs) can protect against acute renal injury induced by ischemia. The mechanism may be the transmission of microRNAs in micro-

capsules, inflammation, anti-oxidation, and anti-apoptotic effects [4-6]. However, whether MSCs-EVs have a protective effect on CsA-induced chronic kidney injury remains unknown In this study, we cultured human umbilical cord mesenchymal stem cells (Human Wharton-Jelly mesenchymal stromal cells, hWJMSCs), and extracted EVs in a conditioned medium, applied them to rat CsA injury model, observed their treatment effect, and tried to study their potential mechanism.

Methods

Ethics statement

In this study, the protocol was approved by the Committee on the Ethics of Animal Experiments of Southeast University. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Cell culture

Fresh human umbilical cords that are usually discarded after delivery were obtained with the written consent of the parents. Healthy human umbilical cords were collected and stored in cold Hank's balanced salt solution (Sigma-Aldrich, USA); cellular isolation was initiated within 4 h. hWJMSC isolation and identification were performed as previously described [6]. After isolation, the MSCs were cultured in low-glucose Dulbecco's modified eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco) at 37°C in a humidified atmosphere with 5% CO_2. Only those cells from the third to sixth passages were used for the experiments. HK-2 cells were obtained from a commercial source (Shanghai Institutes for Biological Sciences, Shanghai, China) and cultured in Low-glucose Dulbecco, Modified Eagle's Medium (DMEM, Gibco BRL, U.S.) containing 3% fetal bovine serum (FBS, Gibco BRL, U.S.) at 37°C in a humidified atmosphere with 5% CO_2.

EVs extraction and characterization

The EVs were obtained from MSC and HFF supernatants as previously described [7]. In brief, the cells were cultured in an FBS-deprived medium and supplemented with 0.5% bovine serum albumin (BSA, Sigma-Aldrich, USA) overnight. The conditioned medium (CM) was collected on day 2. After centrifugation at 2,000 × g for 20 min to remove the debris, the CM was ultra-centrifuged at 100,000 × g for 1 h at 4°C in an SW41 swing rotor (Beckman Coulter Optima L-80K Ultracentrifuge; Beckman Coulter, Fullerton, CA, USA). EVs pellets were suspended in serum free M199. The EVs characterization was performed as previously described [7].

Animal study

Adult (9-11 week old) male SD rats were purchased from Comparative Medicine Center of Yangzhou University (Yangzhou, China). The animals were housed in the animal care facility at the Animal Center of Southeast University, Nanjing, China, and all procedures were performed in accordance with the Animal Care Policies of Southeast University. The animal ethics committee of Southeast University confirmed that our study was approved. The rats were randomized into five treatment groups: in the Sham Group, the rats were fed with a mineral oil vehicle; in the CsA Group, the rats were fed cyclosporine-A (15 mg/kg/day) [8]; in the EVs-therapy group, the rats were fed cyclosporine-A (15 mg/kg/day), and were injected intravenously with MSCs-EVs (100 μg in 1 mL of vehicle) at d7 and d21, respectively. After 1 month, the animals were euthanized by exsanguination under general anesthesia. The left kidney and blood were collected for measurement.

In vitro study

For experiments, HK2 cells were seeded in 5% FBS DMEM at 2.5 × 10^5 cells per well, in 6-well cell culture plates. After 24 h, the culture medium was replaced with 0% FBS, DMEM supplemented with 0.1% bovine serum albumin to arrest cell growth and synchronize the cells. After 12 h in the serum-free medium, the cells were treated with CsA (10 μg/mL) [9] for 24 h. In the MSCs-EVs therapy group, about 50 μg of EVs were added into each well when treated with CsA. 24 h later, the cells were harvested for assay.

Histological examinations

Rats' kidneys were fixed in 4% paraformaldehyde (pH 7.4) and gradually dehydrated, then embedded in paraffin. The samples were cut

into 4-μM sections. Masson's trichrome staining was employed to assess the deposition of collagen in the renal interstitium. The degree of interstitial fibrosis was scored semi-quantitatively [10]. Immunohistochemistry was also carried out. 4 μm-thick sections were labeled with a rabbit antibody to rat α-SMA (dilution 1:500; Abcam, Cambridge, UK). followed by an HRP-conjugated secondary antibody using diaminobenzidine (DAB) reagents as a substrate and then counterstained with hematoxylin. A negative control was performed by omitting primary antibodies. Semi-quantitative scoring was conducted as previously described [11].

Renal function

Serum creatinine was measured using a colorimetric microplate assay based on the Jaffe reaction (BioAssay Systems, Hayward, CA, USA).

ROS assay forHK2 cells

An ROS level assay was conducted as previously described [12, 13]. After EVs or vehicle exposure for 24 h, 10 mM 2',7'-dichlorfluorescein-diacetate (DCFH-DA) was briefly added to the HK2 cells. Cells without injury were also assayed as controls. The cells were incubated for 20 min at 37°C and washed in a serum-free medium thrice to remove extracellular DCFH-DA. The Fluorescence intensity was detected using a fluorescence spectrophotometer at the excitation and emission wavelengths of 488 and 525 nm, respectively.

Western blot analysis

Cytoplasmic proteins were extracted from the HK2 cells. Target proteins were measured through Western blot analysis using rabbit antibodies against α-SMA (dilution 1:800; Abcam, Cambridge, UK). We used an electrochemiluminescence (ECL) chromogenic substrate to visualize the bands, and the intensity of the bands was quantified by densitometry (Quantity One software; Bio-Rad). Antibodies to GAPDH (Santa Cruz) were used as internal control.

MDA and SOD assays

Malondialdehyde (MDA) in the HK2 cells, a product of reduction during lipid peroxidation [14], was detected using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The SOD activity assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) was enrolled. The assays were conducted according to the manufacturer's instructions.

Statistical analysis

The statistical software SPSS (Ver.18.0) was used for the data analysis. A one-way ANOVA with a Sidak post hoc test or a Kruskal-Wallis test with Dunn's posttest was employed to determine the differences in the groups. A value of $P<0.05$ was considered significant.

Results

Characterization of MSCs derived EVs

The hWJMSCs and their EVs were characterized as previously described [15]. The hWJMSC morphology was spindle-shaped and adhered to plastic surfaces. The MSC surface markers (CD44, CD73, CD90, and CD105) were positive, but the expressions of the hematopoietic markers (CD45, CD34 and CD14) and an endothelial marker (CD31) were negative (data not shown). Transmission electron microscopy showed that EVs presented as heterogeneous lipid bilayer vesicles of approximately 30-500 nm in diameter and were characterized as cup-shaped or irregular-shaped (data not shown). A FACS analysis showed that the EVs surface molecules were identical to their original cells (data not shown).

MSCs derived EVs reduced renal fibrosis and saved renal function in CsA-induced renal injury in rats

As shown in **Figure 1A**, renal fibrosis, which was evaluated by Masson's staining, was formed at 4 weeks after the CsA administration. After the EVs therapy, the renal fibrosis was alleviated. The fibrosis score (**Figure 1C**) exhibited the same outcome. In addition, α-SMA staining (**Figure 1B**) was also performed, and it showed that EVs could also mitigate the renal EMT induced by CsA. On the other hand, the serum creatinine levels were more reduced in the EVs therapy group than in the CsA injury group (**Figure 1D**). Taken together, we found MSCs derived EVs reduced renal fibrosis and reversed the EMT process induced by CsA as well as saved renal function.

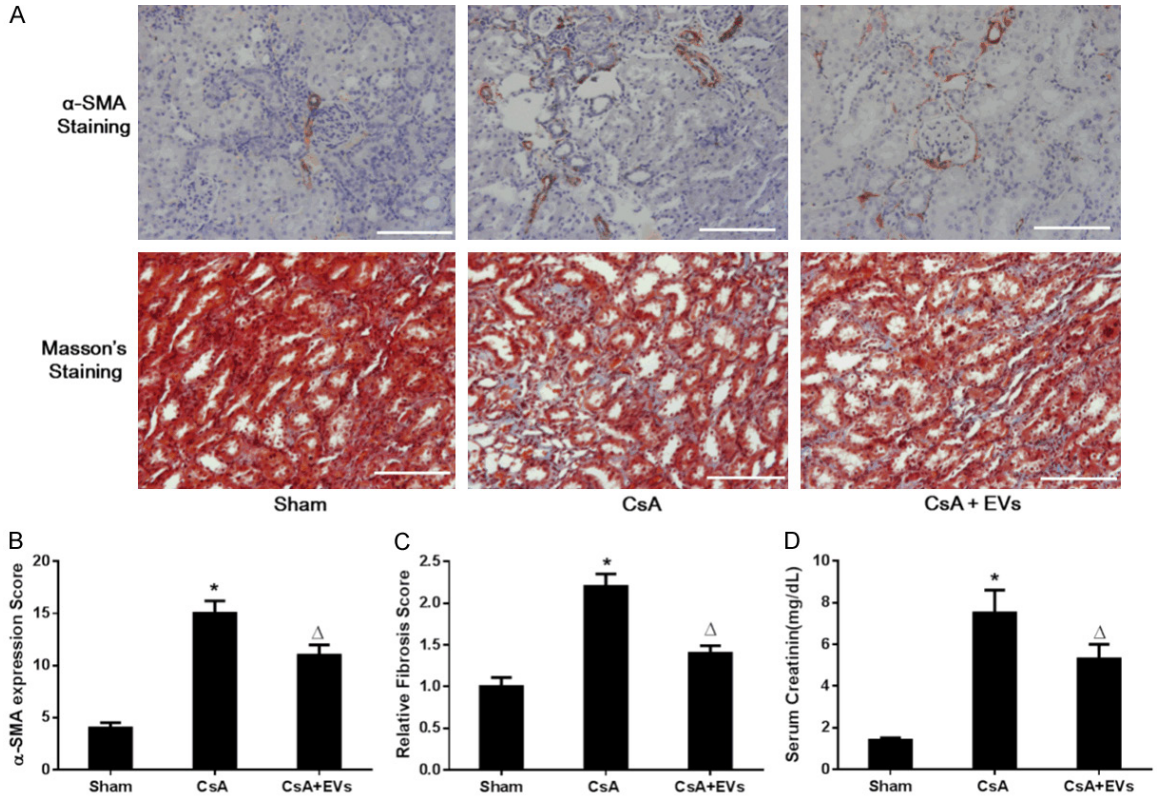

Figure 1. MSCs-EVs alleviated CsA-induced renal fibrosis and saved renal function in Rats. A. α-SMA staining and Masson's trichrome staining for rats' renal tissues in the sham group, the CsA group and the EVs-therapy group. B. The α-SMA expression score in each group. C. The relative renal fibrosis score in each group. D. Serum creatinine in each group. (Sham, sham group; CsA, CsA Group; CsA+Evs, MSCs-EVs treated group; *P<0.05 vs Sham, ΔP<0.05 vs CsA, Bar length, 50 μm).

MSCs-EVs abrogated the EMT process and reduced ROS generation in the CsA injury HK2 model

In order to study the potential mechanism, we set up the HK2 injury model by CsA. After CsA treatment, α-SMA expression, which is a marker of EMT, became elevated. Interestingly, it is observed that α-SMA was down-regulated in the MSCs-EVs treated group (**Figure 2A**). In the meantime, we measured the ROS Level of the HK2 cells using the DCFHDA method and found that hWJMSCs-EVs could also reduce the ROS levels in CsA injured HK2 cells (**Figure 2B**).

The oxidative stress was alleviated by MSCs-EVs treating CsA injured HK2 cells

We further evaluated the SOD and MDA levels, which are regarded as oxidative stress markers. It was observed that the SOD level was reduced after injury while the EVs from MSCs resorted to the SOD level (**Figure 3A**). The MDA level was more reduced in the MSCs-EVs treatment group than in the CsA treatment group (**Figure 3B**).

Discussion

In present study, we isolated EVs from MSCs and applied them into a CsA injury animal model by intravenous injection at 7 d and 14 d respectively. The results showed that renal fibrosis was alleviated and the EMT process was abrogated in the EVs treatment group. *in vitro*, and it was also found that EVs treatment could suppress oxidative stress and abrogate EMT in CsA injured HK2 cells.

Our previous studies demonstrated that MSCs-EVs could protect against acute kidney injury by its anti-oxidative and anti-inflammatory effects. The potential mechanism may be miRNA or a functional protein cargo by EVs [4-6, 16, 17]. However, it is still unknown if there is protective effect of EVs for renal chronic injury. CsA-

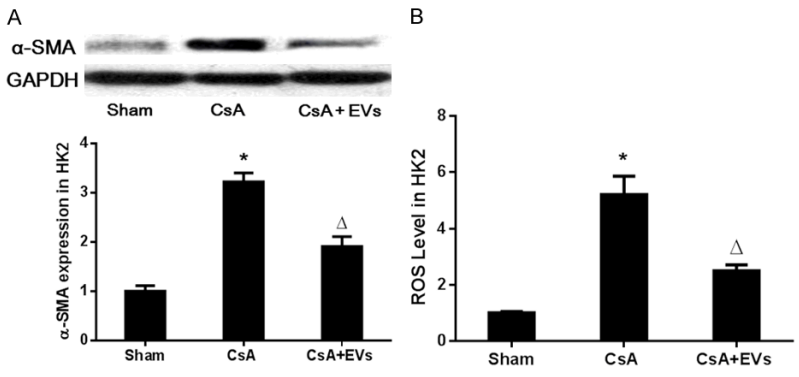

Figure 2. MSCs-EVs reduced α-SMA expression and ROS production in CsA injured HK2 cells. A. Western-blot analysis for α-SMA expression in HK2 cells in the sham group, the CsA group, and the EVs-therapy group. B. The ROS level in the HK2 cells in each group. (Sham, sham group; CsA, CsA group; CsA+EVs, MSCs-EVs treated group; *P<0.05 vs Sham, ΔP<0.05 vs CsA).

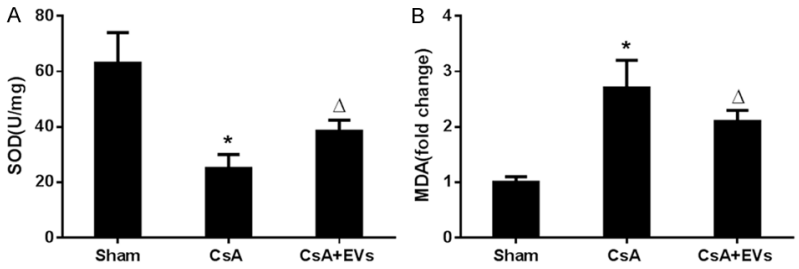

Figure 3. MSCs-EVs alleviated oxidative stress in the CsA injured HK2 cells. A. SOD concentration in in the HK2 cells in the sham group, the CsA group, and the EVs-therapy group. B. The MDA level in the HK2 cells in each group. (Sham, sham group; CsA, CsA group; CsA+Evs, MSCs-EVs treated group; *P<0.05 vs Sham, ΔP<0.05 vs CsA).

induced renal injuries are observed after kidney transplantation in the clinic, which troubles the clinicians, as discontinuation may lead to immune rejection, and there is no ideal solution for this problem. The present pre-clinical study suggested a novel potential way to solve it. On the other hand, a single administration of MSC-EVs was conducted for AKI in our previous studies. However, in the present study, two EVs injections were carried out at 7 d and 14 d respectively in which CsA-induced renal fibrosis was a chronic injury. The novel EVs administration method was used to enhance the therapeutic effects. The potential problem is that there may be an inter-species immune cross-reactivity as EVs produced by human cells are injected into the rat twice. However, studies have also revealed that microcapsules produced by mesenchymal stem cells exhibit lower antigenicity [18].

We further investigated the preliminary mechanism. It was proved previously that MSCs-derived EVs could take an anti-oxidative role in the AKI model and the mechanism may be that the MSC-EVs enhanced the Nrf2/ARE pathway or suppressed NOX2 expression, which could produce reactive oxygen species (ROS) [5, 6]. In the present study, we also found MSC-EVs could also alleviate oxidative stress. On the other hand, it was suggested that oxidative stress might play an essential role in CsA-induced nephrotoxicity [19, 20]. NOX2 was also regarded as a key molecule mediating TGF-β signaling in this process [21]. Thus, we speculate that NOX2 suppression by MSC-EVs might be the underlying mechanism of the protection for CsA-induced nephrotoxicity. We would confirm it in a future study.

In summary, we observed that CsA-induced nephrotoxicity could be alleviated by MSCs-EVs in a rat model, and we found that the anti-oxidative property of MSCs-EVs might be the mechanism. However, the detailed mechanisms should be further investigated.

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Disclosure of conflict of interest

None.

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