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Basic Study

Synergism of calycosin and bone marrow-derived mesenchymal stem cells to combat podocyte apoptosis to alleviate adriamycin-induced focal segmental glomerulosclerosis

Qiong-Dan Hu, Rui-Zhi Tan, Yuan-Xia Zou, Jian-Chun Li, Jun-Ming Fan, Fahsai Kantawong, Li Wang

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Abstract

BACKGROUND

Bone marrow-derived mesenchymal stem cells (MSCs) show podocyte-protective effects in chronic kidney disease. Calycosin (CA), a phytoestrogen, is isolated from *Astragalus membranaceus* with a kidney-tonifying effect. CA preconditioning enhances the protective effect of MSCs against renal fibrosis in mice with unilateral ureteral occlusion. However, the protective effect and underlying mechanism of CA-pretreated MSCs (MSCs^{CA}) on podocytes in adriamycin (ADR)-induced focal segmental glomerulosclerosis (FSGS) mice remain unclear.

AIM

To investigate whether CA enhances the role of MSCs in protecting against podocyte injury induced by ADR and the possible mechanism involved.

METHODS

ADR was used to induce FSGS in mice, and MSCs, CA, or MSCs^{CA} were administered to mice. Their protective effect and possible mechanism of action on podocytes were observed by Western blot, immunohistochemistry, immunofluorescence, and real-time polymerase chain reaction. *In vitro*, ADR was used to stimulate mouse podocytes (MPC5) to induce injury, and the supernatants from MSC-, CA-, or MSCs^{CA}-treated cells were collected to observe their protective effects on podocytes. Subsequently, the apoptosis of podocytes was detected *in vivo* and *in vitro* by Western blot, TUNEL assay, and immunofluorescence. Overexpression of Smad3, which is involved in apoptosis, was then induced to evaluate whether the MSCs^{CA}-mediated podocyte protective effect is associated with Smad3 inhibition in MPC5 cells.

RESULTS

CA-pretreated MSCs enhanced the protective effect of MSCs against podocyte injury and the ability to inhibit podocyte apoptosis in ADR-induced FSGS mice and MPC5 cells. Expression of p-Smad3 was upregulated in mice with ADR-induced FSGS and MPC5 cells, which was reversed by MSC^{CA} treatment more significantly than by MSCs or CA alone. When Smad3 was overexpressed in MPC5 cells, MSCs^{CA} could not fulfill their potential to inhibit podocyte apoptosis.

CONCLUSION

MSCs^{CA} enhance the protection of MSCs against ADR-induced podocyte apoptosis. The underlying mechanism may be related to MSCs^{CA}-targeted inhibition of p-Smad3 in podocytes.

Key Words: Calycosin; Mesenchymal stem cells; Focal segmental glomerulosclerosis; Apoptosis; Smad3

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Core Tip: Calycosin (CA)-pretreated mesenchymal stem cells (MSCs^{CA}) enhanced the protective effect of MSCs against adriamycin (ADR)-induced podocyte injury *in vitro* and *in vivo* by inhibiting apoptosis, accompanied by more reversal of the upregulated expression of p-Smad3 after ADR induction. Smad3 overexpression eliminated the inhibitory effect of MSCs^{CA} on podocyte apoptosis, suggesting that MSCs^{CA} inhibit podocyte apoptosis by targeting p-Smad3. These results broaden our understanding of the potential of MSCs pretreated with herbal extract and provide new theories for possible therapeutic mechanisms for ADR-induced focal segmental glomerulosclerosis.

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INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) is the most common primary glomerulopathy and the dominant pathological type of chronic kidney disease (CKD)[1,2], associated with high albuminuria and end-stage renal disease (ESRD) with a poor prognosis[3,4]. FSGS is linked with injury or even depletion of podocytes, manifested by the gradual disappearance of podocyte-specific markers such as podocin[5, 6]. As podocyte injury plays a critical role in FSGS progression, protecting podocytes is promising to prevent ESRD in patients with FSGS[7].

Apoptosis of podocytes has been widely studied in previous studies[8-10], and inhibition of podocyte apoptosis has been reported to delay FSGS progression[11]. Podocyte apoptosis is characterized by the loss of Bcl-2 protein and the increase of Bax protein[12,13]. Recently, Smad3-related pathways have been reported to be involved in podocyte apoptosis[14]. However, the underlying mechanism remains unclear, and no specific effective treatment can prevent podocyte apoptosis.

Mesenchymal stem cells (MSCs) are multipotent stem cells that exhibit varying potential for multilineage cell differentiation as well as the capacity for self-renewal[15]. Therefore, using MSCs to treat various diseases is worth exploring[16-18]. MSCs treat diabetic nephropathy by protecting podocytes[19-21], and bone marrow-derived MSC (BMSC) transplantation can attenuate FSGS progression in a rat model of FSGS[22,23]. In addition, the protective effects of MSC derivatives or exosomes on podocytes have also been reported[24,25]. However, the application of MSCs is also

limited. For instance, MSCs may be losing their biological function after being isolated and cultured for a long time. After infusion, MSCs must face harsh environments with various stressors such as inflammation, hypoxia, high acidity, or reduced energy reserve. On this account, preconditioning, genetic modification, and delivering MSCs with biomaterials have been developed[26]. Thus, it is important to explore how MSCs can overcome adverse microenvironments to enhance their therapeutic benefits.

Calycosin (CA), a phytoestrogen with a kidney-tonifying effect, is isolated from *Astragalus membranaceus*. It has been reported that CA is the top component of potentially active compounds for the treatment of nephrotic syndrome[27]. Moreover, CA has also been found to be an active ingredient in the treatment of adriamycin (ADR) nephropathy using network pharmacology combined with transcriptomics[28]. Our research group used CA-pretreated MSCs to treat mice with unilateral ureteral occlusion (UUO) and found that they improved renal fibrosis and inhibited necrosis of renal tubular epithelial cells more than normal MSCs did[29]. However, the protective effect on podocytes and their mechanism of action remain unknown.

In rodents, ADR can induce rapid podocyte injury characterized by massive foot process effacement and glomerulosclerosis, which serves as a model of FSGS[30,31]. In the present study, we compared the antiapoptotic efficacy of CA-pretreated MSCs (MSCs^{CA}) to that of MSCs or CA in a mouse model of FSGS induced with ADR and *in vitro*, as well as the possible mechanisms of action involved.

MATERIALS AND METHODS

Animal experiments

The C57BL/6 mice utilized in this investigation were bought from Chengdu Dashuo Biotechnology Co., LTD. in China. They were male, 8 wk old, and weighed 22-25 g. All the mice were kept in a specific disease-free space with 12 h of light and dark cycles and had free access to water and food. The mice were randomly divided into the following six groups: Normal control group; ADR injection group; ADR with Dulbecco's modified Eagle's medium (DMEM; 200 μ L) injection (ADR + DMEM); ADR with 200 μ L MSCs (10^6 cells/mL) (ADR + MSCs); ADR with 200 μ g/mL CA (10^6 cells/mL) (ADR + CA); and ADR with MSCs preconditioned with 200 μ g/mL CA (10^6 cells/mL) (ADR + MSCs^{CA}). For ADR-induced FSGS, the mice were injected with 10 mg/kg ADR (Shenzhen Main Luck Pharmaceuticals Inc.) *via* the tail vein. The normal control mice were injected with vehicle (saline). MSCs, CA dissolved in DMEM, and MSCs^{CA} were injected *via* the tail vein 4 wk after ADR injection once weekly. Since both MSCs and CA are soluble in DMEM, mice in the ADR + DMEM group were given an equal volume of normal DMEM as the solvent control. All mice were killed at 8 wk after ADR injection. All animal experiments were carried out in accordance with the recommendations of the Institute of Nutrition and Health's Animal Care and Utilization Committee, and were approved by the Southwest Medical University's Animal Ethics Committee (No. 20210223-024).

Isolation of MSCs

As previously described, MSCs were isolated from the leg bone marrow of male C57BL/6 mice aged 6-8 wk[32]. Briefly, cells were grown at 37 °C and 5% CO₂ in DMEM Petri plates with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, United States), 1 g/L glucose, and 1% penicillin-streptomycin (Beyotime, Shanghai, China). At 24 h, the medium was changed to remove the non-adherent cells. The MSCs were passed once 90% confluence was reached. As described previously[26], anti-CD29 (102205; Biolegend), anti-CD90 (ab24904; Abcam, Cambridge, MA, United States), and anti-CD11b (101205; Biolegend, San Diego, CA, United States) antibodies were used to label MSCs, and the purity of the MSCs was analyzed using a BD FACSVerser (Becton, Dickinson and Company, Franklin Lakes, NJ, United States).

CA pretreatment of MSCs

CA ($\geq 94\%$ purity) was purchased from Cayman Chemical Company (Ann Arbor, MI, United States). The stoste used for MSC pretreatment included full medium and CA (200 g/mL) dissolved in DMEM as previously described[29]. After incubation for 72 h, the MSCs and MSCs^{CA} were injected into mice, and the supernatants were used to treat mouse podocytes (MPC5) for 48 h.

Urine albumin-creatinine ratio

Random urine samples were collected, followed by determining the albumin concentration with a mouse albumin ELISA kit (Sangon Biotech, China) and creatinine with a creatinine assay kit (Nanjing Jiancheng, Jiangsu Province, China). The urine albumin-creatinine ratio was calculated by dividing the urine albumin concentration by the creatinine concentration.

Hematoxylin-eosin staining

Mouse kidneys were fixed in 4% neutral formaldehyde followed by paraffin embedding. The paraffin sections were rehydrated in a graded ethanol series and subjected to hematoxylin-eosin (HE) staining

(Beyotime, Shanghai, China) as previously described[33].

Immunohistochemistry

The sections underwent antigen retrieval in 0.01 M citric acid solution (pH 6.0) in a microwave oven for 10 min after deparaffinization and rehydration. To inhibit endogenous peroxidase, the slices were incubated with 5% H₂O₂ for 15 min. The sections were then further blocked for 30 min at room temperature with 5% bovine serum albumin (BSA), and then incubated overnight at 4 °C with anti-p-Smad3 antibody (C25A9; Cell Signaling Technology, Danvers, MA, United States). The slices were treated with secondary antibodies for 1 h at room temperature following PBS washing. Images were recorded with a light microscope (Eclipse 80i; Nikon, Japan).

TUNEL assay

TUNEL assay was used to evaluate podocyte apoptosis in the kidneys after ADR induction, as previously described[34]. After the mouse kidneys were fixed in 4% neutral formaldehyde followed by paraffin embedding, the paraffin sections were used for staining. Podocyte apoptosis was measured through the utilization of a One-step TUNEL In Situ Apoptosis Assay Kit (AF488; Green) (E-CK-A321; Elabscience, China). The images were captured with an orthotopic fluorescence microscope (DM4B; Leica, Germany).

Cell culture and treatment

Prof. San-Tao Ou (Department of Nephrology, Southwest Medical University) kindly donated the conditionally immortalized MPC5 cell line. The cells were grown at 33 °C in RPMI-1640 medium supplemented with 10 IU/mL recombinant interferon and 10% FBS. After the MPC5 cells were cultured at 37 °C for 14 d to induce differentiation, the differentiated cells were treated with different concentrations of ADR for 24 h. A Smad3 overexpressing MPC5 cell line was established with the Smad3 overexpression plasmid pcDNA3.1-Smad3 which was described previously[35].

Immunofluorescence

After treatment, the MPC5 cells or frozen sections were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.25% Triton X-100 (in PBS), and blocked with 5% BSA for immunofluorescence. After that, the frozen sections or MPC5 cells were incubated with anti-podocin (BA0290; Boster, Wuhan, China), anti-Bax (AF0120; Affinity, United States), and anti-Bcl-2 (AF6139; Affinity) antibodies at 4 °C overnight. After washing with PBS, the frozen sections or MPC5 cells were incubated with Alexa Fluor 594 Donkey anti-mouse/rabbit secondary antibodies (Thermo Fisher Scientific, Waltham, MA, United States) for 1 h at room temperature. The nuclei were stained with 4',6-diamidino-2-phenylindole (Sangon Biotech). Images were captured with a fluorescence microscope (EVOS FL Auto, Thermo Fisher Scientific, United States).

Real-time quantitative polymerase chain reaction

TRIzol reagent (Invitrogen, Carlsbad, CA, United States) was used to separate total RNA from cells or kidneys, and a Reverse Transcription Kit (Promega, Madison, WI, United States) was used to obtain cDNA. Using Master Mixture (TaKaRa, Dalian, China) and LightCycler 480 equipment (Roche, Germany), the podocin mRNA expression levels were assessed. The internal control used was GAPDH. Using 2^{-ΔΔCt} analysis, the relative expression of the target gene was standardized to GAPDH expression. The primer sequences used are shown in [Supplementary Table 1](#).

Western blot analysis

Using RIPA lysis buffer (Beyotime), total proteins were extracted from kidneys or cells. The protein concentrations were determined with a BCA protein assay kit (Beyotime). Proteins were transferred onto polyvinylidene difluoride membranes after being separated by 12% SDS-PAGE. Then the membranes were incubated with anti-podocin (BA0290; Boster), anti-p-Smad3 (C25A9; Cell Signaling Technology), anti-Smad3 (C67H9; Cell Signaling Technology), anti-Bax (AF0120; Affinity), anti-Bcl-2 (AF6139; Affinity), and anti-GAPDH (AB0037; Abways, China) antibodies at 4°C overnight. The membranes were treated with the relevant secondary antibody at room temperature for 1 h after being rinsed with Tris-buffered saline with Tween (TBST). The protein bands were depicted with an enhanced ECL kit (Boster) and a chemiluminescence imaging system (ChemiScope 6200; Clinx, China). ImageJ software (NIH, Bethesda, MD, United States) was used to calculate the band gray intensity.

Apoptosis detection by flow cytometry

The cells were digested with trypsin-EDTA solution (C0201; Beyotime), collected in a centrifuge tube, and centrifuged for 5 min at 1800 rpm, and the supernatant was discarded. The cells were resuspended with 1 mL precooled PBS. According to the Annexin V-FITC/PI Apoptosis Detection kit's instructions (Vazyme, Nanjing, China), the prepared propyl iodide staining solution was added to the cells and incubated at 37 °C for 10 min without light. Red fluorescence was detected at an excitation wavelength of 488 nm and light scattering was detected with a BD FACSVerser (Becton, Dickinson).

Statistical analysis

The mean and standard deviation of the data are displayed. Using SPSS 21.0 software (IBM Corp., Chicago, IL, United States), one-way analysis of variance was used to compare the data. $P < 0.05$ was considered statistically significant.

RESULTS

MSCs^{CA} enhance the protective effect of MSCs on podocyte injury in ADR-induced FSGS

To investigate whether CA pretreatment enhances the protective effect of MSCs on podocyte injury in ADR-induced FSGS mice, we treated mice with MSCs, CA, or MSCs^{CA}. Due to MSCs, CA, and MSCs^{CA} being dissolved in DMEM, a DMEM group was separately designed as the solvent control group to exclude the protective effect of DMEM-containing nutrients on podocytes (Figure 1A). The identification of MSCs, the chemical formula of CA, and its appropriate concentration can be found in our previous research[26]. Eight weeks after ADR injection, increased urinary albumin excretion was detected in ADR-treated mice, and MSCs^{CA} reversed this more significantly than MSCs or CA alone. However, there was no difference between the DMEM group and the model group (Figure 1B). HE staining showed that glomerular atrophy and FSGS were prominent in the ADR and DMEM groups, but MSCs^{CA} treatment reversed this change and was superior to MSCs and CA treatment (Figure 1C). Immunostaining, real-time quantitative polymerase chain reaction (RT-PCR), and Western blot analysis showed that the expression of podocin, a podocyte-specific marker, was significantly reduced in the ADR and DMEM groups; however, MSCs^{CA} treatment best restored its expression (Figure 1D-G). The above evidence indicated that MSCs^{CA} treatment better protected podocytes from ADR injury in FSGS mice.

The capacity of MSCs to prevent apoptosis in ADR-induced FSGS is improved by CA pretreatment

To determine the effect of MSCs^{CA} on renal cell apoptosis, Western blot and TUNEL assay were performed. Expression of Bax protein as an apoptosis marker was significantly increased in the ADR and DMEM groups compared with the normal group, and their levels were reduced after MSC or CA treatment (Figure 2A and B). MSCs^{CA} reduced ADR-induced Bax protein expression more significantly than MSCs or CA. The changing trend in Bcl-2 protein expression was opposite to that of Bax in each group (Figure 2A and C). TUNEL assay showed an obvious increase in the brightness and range of green fluorescence in the ADR group, which was weakened by MSCs^{CA} treatment (Figure 2D). The above data indicated that MSCs^{CA} enhanced the antiapoptotic effect of MSCs on kidney cells of ADR-induced FSGS mice.

P-Smad3 is upregulated in podocytes of ADR-induced FSGS mice and reversed after MSCs^{CA} treatment

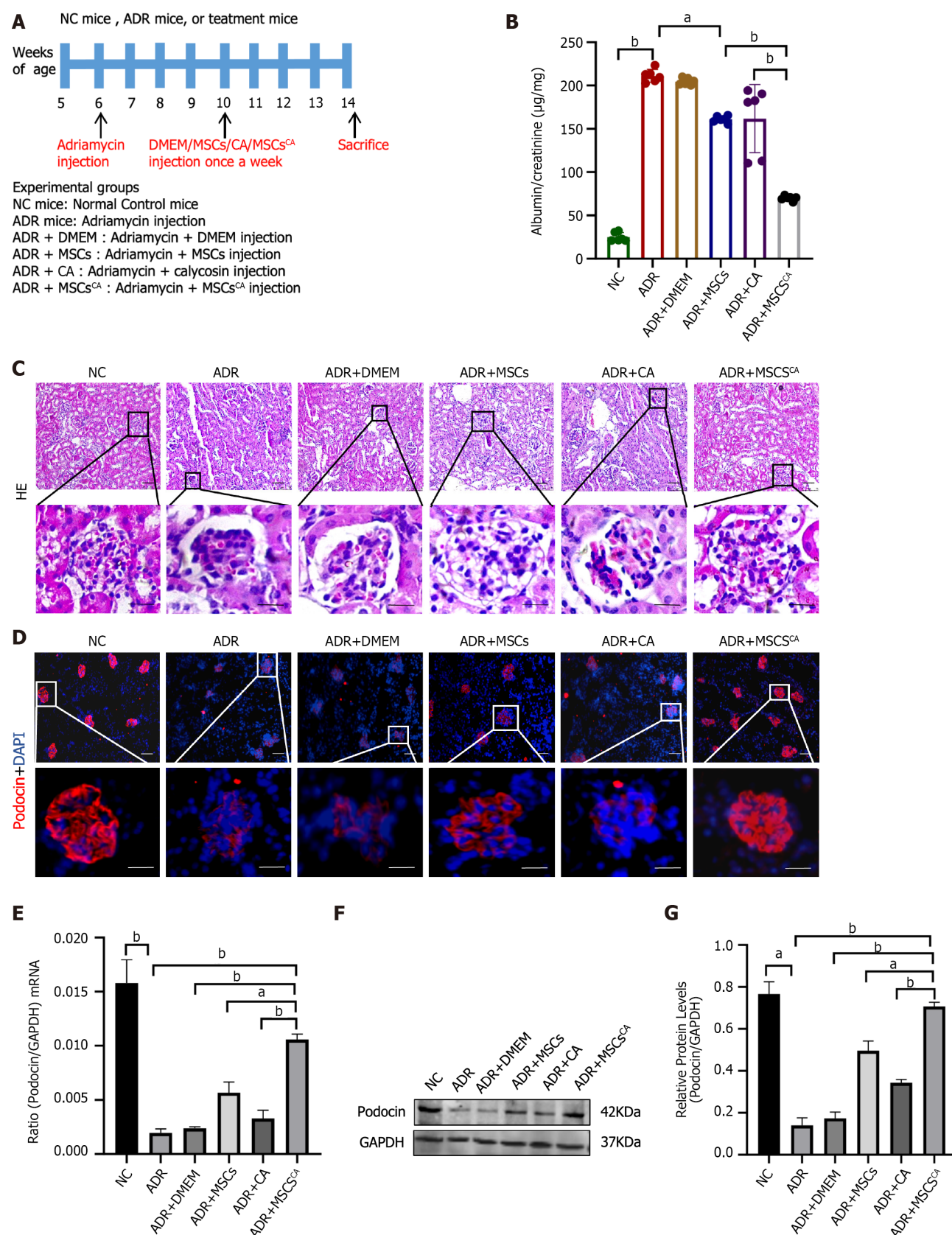
It has been reported that the Smad3 protein is involved in podocyte apoptosis[13], so we examined the effect of MSCs^{CA} on the expression of Smad3 and p-Smad3 proteins. As expected, we found by Western blot and immunohistochemistry that MSCs^{CA} treatment significantly reversed the upregulation of p-Smad3 in ADR-treated mouse renal podocytes, and the effect was superior to that of MSC and CA treatment (Figure 3). The above evidence suggested that p-Smad3 was involved in the ADR-induced injury of podocytes and the recovery after MSCs^{CA} treatment.

The capacity of MSCs to reduce the injury caused by ADR-stimulated MPC5 cells in vitro is improved by CA pretreatment

To further demonstrate the enhanced potential of MSCs^{CA} to protect podocytes from ADR injury, we cultured and treated MPC5 cells. Immunofluorescence staining, RT-PCR, and Western blot showed that 1.2 μ M/mL ADR decreased the expression of podocin mRNA and protein, while the expression was significantly promoted by treatment with conditioned medium from MSCs, or CA. Importantly, conditioned medium from MSCs^{CA} further elevated the expression of podocin mRNA and protein compared with the ADR group (Figure 4). Therefore, MSCs^{CA} protected podocytes from ADR injury better than MSCs or CA alone.

CA pretreatment enhances the ability of MSCs to inhibit apoptosis in ADR-stimulated MPC5 cells

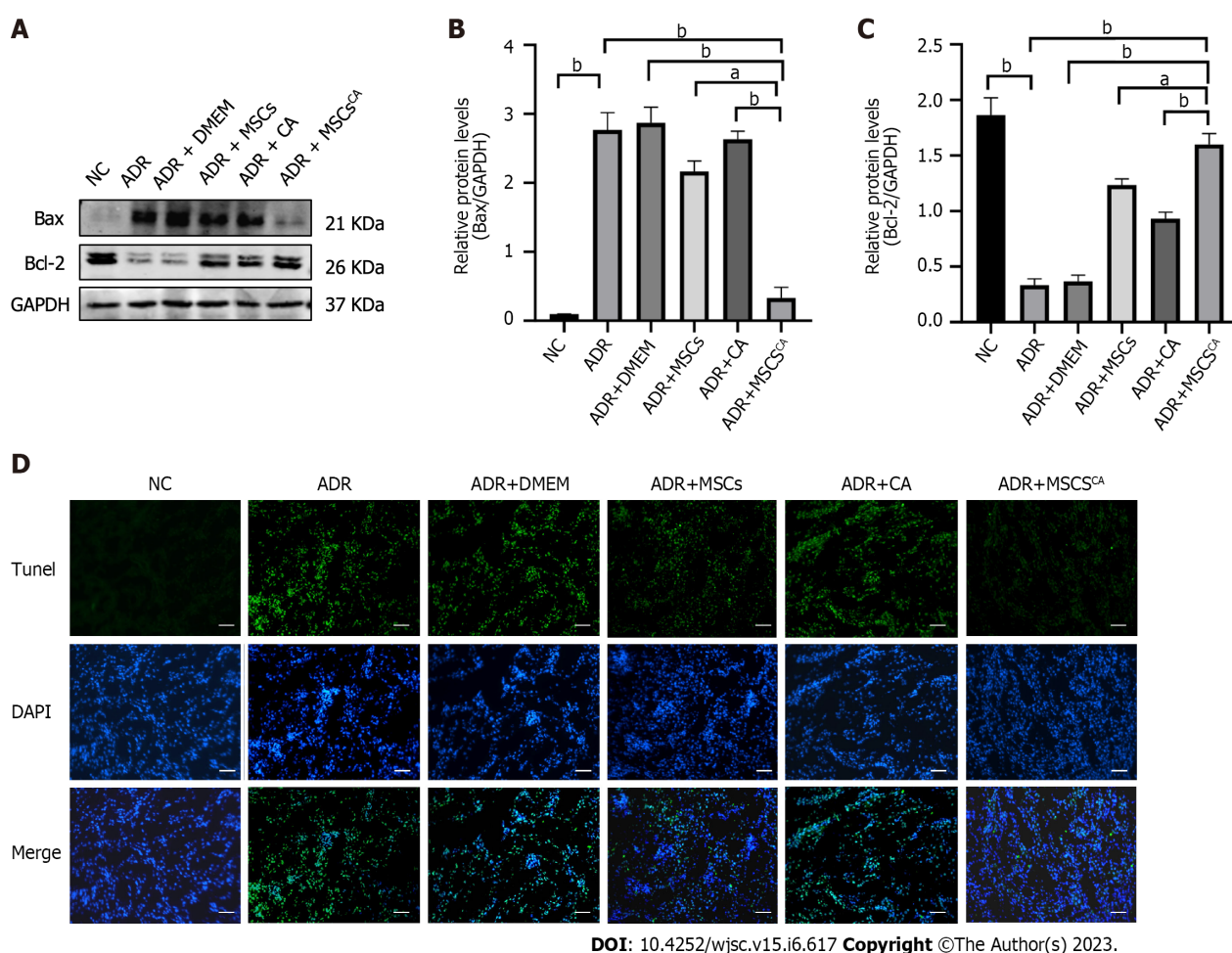
Immunofluorescence staining and Western blot demonstrated that the protein level of Bax was upregulated in MPC5 cells treated with ADR, but its expression was significantly inhibited by treatment with a conditioned medium from MSCs, or CA alone (Figure 5). The effects of the conditioned medium from MSCs^{CA} were more pronounced. The trend for Bcl-2 protein expression was the opposite. These findings revealed that Ca-pretreated MSCs enhanced the inhibitory effect of MSCs on podocyte apoptosis.



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Figure 1 Mesenchymal stem cells pretreated with calycosin enhance the protective effect of mesenchymal stem cells on podocyte injury in adriamycin-induced focal segmental glomerulosclerosis mice. A: Mice received adriamycin injections through the tail vein at week 6, were injected with Dulbecco's modified eagle medium, mesenchymal stem cells (MSCs), calycosin (CA), and MSCs pretreated with CA (MSCs^{CA}) at week 10, respectively, and were sacrificed at week 14; B: Levels of albumin/creatinine ratio in urine ($n = 6$), $^aP < 0.05$, $^bP < 0.001$; C: Pathological changes in the kidneys of mice examined by hematoxylin-eosin staining. Typical glomeruli are indicated by black boxes and enlarged to the next row. Bar = 50 μm; D: Changes in the kidneys of mice examined by

podocin immunofluorescence staining. Glomeruli are indicated by white boxes and enlarged to the next row. Bar = 50 μ m; E: Relative (podocin/GAPDH) mRNA expression analyzed by real-time quantitative polymerase chain reaction. Data are expressed as the mean $\pm$ SD ($n = 6$). ^a $P < 0.05$, ^b $P < 0.001$; F and G: Relative protein levels (podocin/GAPDH) detected by Western blot. Data are expressed as the mean $\pm$ SD ($n = 3$). ^a $P < 0.05$, ^b $P < 0.001$. NC: Normal control; ADR: Adriamycin; DMEM: Dulbecco's modified eagle medium; MSCs: Mesenchymal stem cells; CA: Calycosin; MSCs^{CA}: Mesenchymal stem cells pretreated with calycosin.

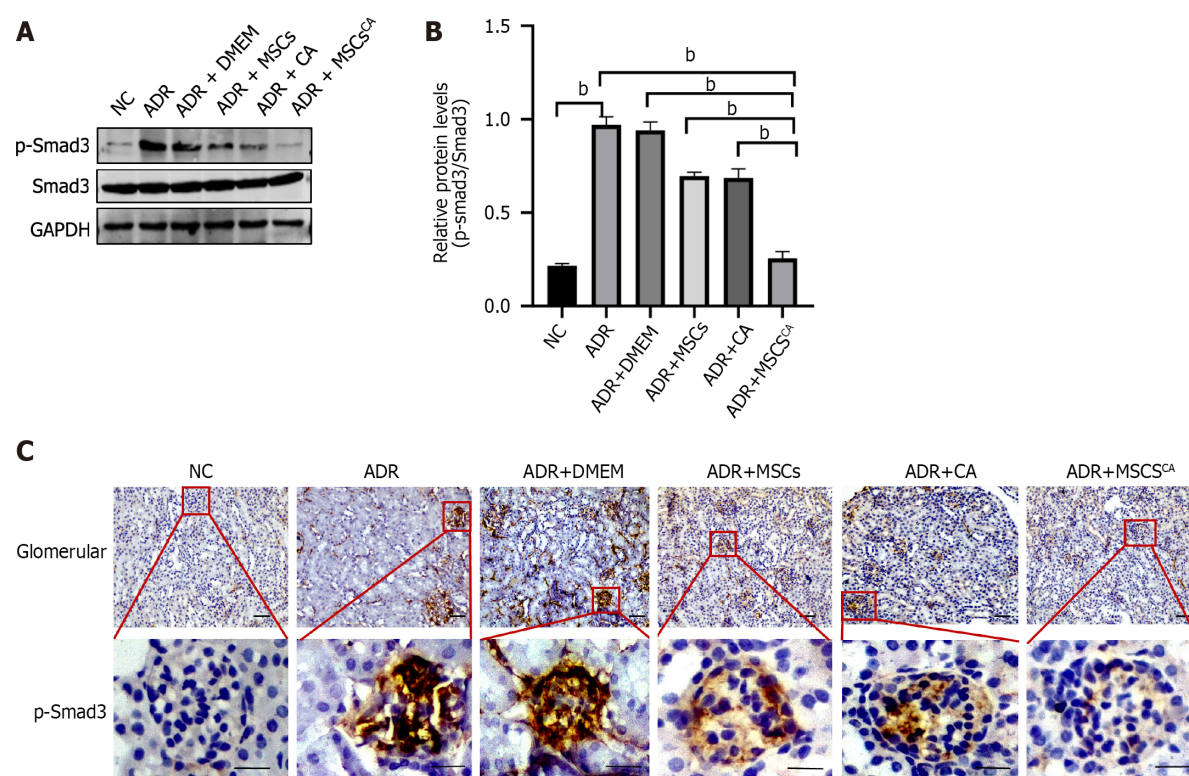


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Figure 2 Calycosin pretreatment enhances the ability of mesenchymal stem cells to inhibit apoptosis in adriamycin-induced focal segmental glomerulosclerosis mice. A-C: Protein expression levels of Bax and Bcl-2 in the kidneys measured by Western blot and normalized to control. Data are expressed as the mean $\pm$ SD ($n = 3$). ^a $P < 0.05$, ^b $P < 0.001$; D: Apoptosis in each group as determined by TUNEL assay. Bar = 50 μ m. NC: Normal control; ADR: Adriamycin; MSCs: Mesenchymal stem cells; CA: Calycosin; MSCs^{CA}: Mesenchymal stem cells pretreated with calycosin.

MSCs^{CA} improve ADR-induced podocyte apoptosis by targeting p-Smad3 expression

As described previously, p-Smad3 is involved in ADR-induced FSGS mice. Further experiments were conducted to explore whether MSCs^{CA} inhibit podocyte apoptosis by targeting p-Smad3. Expression of p-Smad3 in MPC5 cells was markedly elevated by ADR stimulation and subsequently significantly downregulated by MSCs^{CA} treatment. The upregulated expression of p-Smad3 was also reversed by MSCs or CA, but to a lesser extent (Figure 6A and B). When Smad3 accompanied by p-Smad3 in MPC5 cells was overexpressed, Bax protein expression was upregulated but Bcl-2 protein expression was downregulated. Meanwhile, MSCs^{CA} treatment no longer showed a protective effect against ADR-induced podocyte apoptosis compared with the group without Smad3 overexpression (Figure 6C-G). Flow cytometry was used to detect apoptosis, and it was found that the apoptosis rate of MPC5 cells was significantly increased after ADR induction compared with the normal group, and MSCs^{CA} reversed this increase. However, after overexpression of Smad3, the apoptosis rate was increased compared with the normal group and the model group regardless of whether MSCs^{CA} were administered. This means that treatment with MSCs^{CA} did not improve the apoptosis of podocytes with Smad3 overexpression (Figure 6H-L). The graphical abstract (created in BioRender.com) is shown in Figure 6M. The above evidence suggested that MSCs^{CA} improved podocyte apoptosis through targeted inhibition of p-Smad3.



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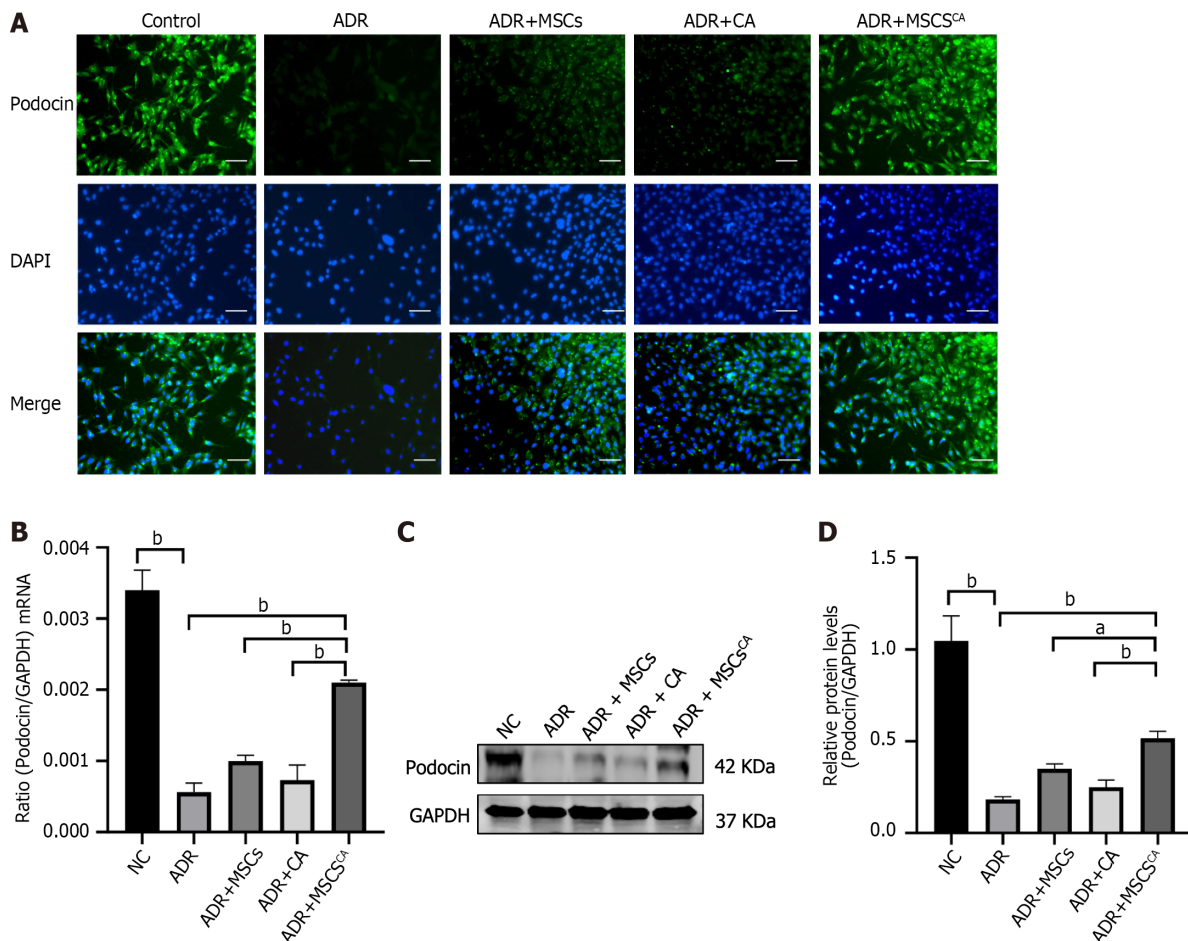
Figure 3 P-Smad3 is upregulated in podocytes of adriamycin-induced focal segmental glomerulosclerosis mice and reversed after treatment with mesenchymal stem cells pretreated with calycosin. A and B: Protein expression levels of p-Smad3 and Smad3 detected using Western blot and normalized to control. Data are expressed as the mean $\pm$ SD ($n = 3$). ^b $P < 0.001$; C: Immunohistochemistry staining for p-Smad3 in mouse glomeruli, which are indicated by red boxes and enlarged to the next row. Bar = 50 μ m. NC: Normal control; ADR: Adriamycin; MSCs: Mesenchymal stem cells; CA: Calycosin; MSCs^{CA}: Mesenchymal stem cells pretreated with calycosin.

DISCUSSION

Increasing evidence has shown that MSCs and derived extracellular vesicles can ameliorate renal deterioration in CKD[36,37]. However, because of a hostile environment with several stresses such as inflammation, high acidity, hypoxia, and depleted energy reserve, few MSCs survive *in vivo* after intravenous or direct local injection[38-40]. The question of whether preconditioning BMSCs can shield them from the damaging environment at the injury site and enhance their functionality has drawn more attention in research. These pretreatments involve the application of supportive materials, cytokines, and natural or synthetic chemicals[41-44]. Researchers have been investigating the preconditioning of MSCs using Chinese herbal medicine or its primary monomer components. There is evidence that resveratrol-pretreated adipose-derived stem cells show increased regenerative capacity in a rat model of diabetes-induced cardiomyopathy[45]. Further research has shown that preconditioning MSCs obtained from umbilical cords with the active ingredient of a Chinese herb, triptolide, primed MSCs to be activated and inhibited the immune response before being delivered[46]. Previous results from our group have also shown that CA-pretreated BMSCs show enhanced antifibrotic activity in UO mice and inhibit tubular epithelial cell necrosis[29]. Therefore, we investigated whether MSCs^{CA} enhance podocyte protection. Similar to previous studies, MSCs^{CA} protected podocytes from ADR-induced apoptosis, both *in vivo* and *in vitro*, which means that they may be a potential therapy for FSGS.

CA is the top ingredient in *Astragalus*, which is one of the most widely used herbs in Chinese medicine to treat kidney disease[47-49]. The effectiveness of CA in CKD has been confirmed in recent years[50-52]. However, whether its combination with MSCs can enhance their efficacy in treating CKD remains to be seen. It has been shown that human MSCs are stimulated to enhance osteogenesis and mineralization by CA-7-O-glucoside obtained from *Astragalus membranaceus*[53]. This result gave us confidence and we also identified the advantages and potential of MSCs^{CA} in the treatment of FSGS, which extends the application of CA and MSCs in FSGS.

The main pathological manifestations of FSGS are podocyte injury and the therapeutic options for FSGS are limited, requiring further research and exploration. Therefore, we explored the mechanism of podocyte injury. Podocyte apoptosis is the main type of podocyte injury, which includes podocyte dedifferentiation, autophagy, and epithelial-mesenchymal transformation[54]. Podocyte apoptosis is caused by many factors, including drugs, infection, and immune disorders[55-57]. ADR is one of the



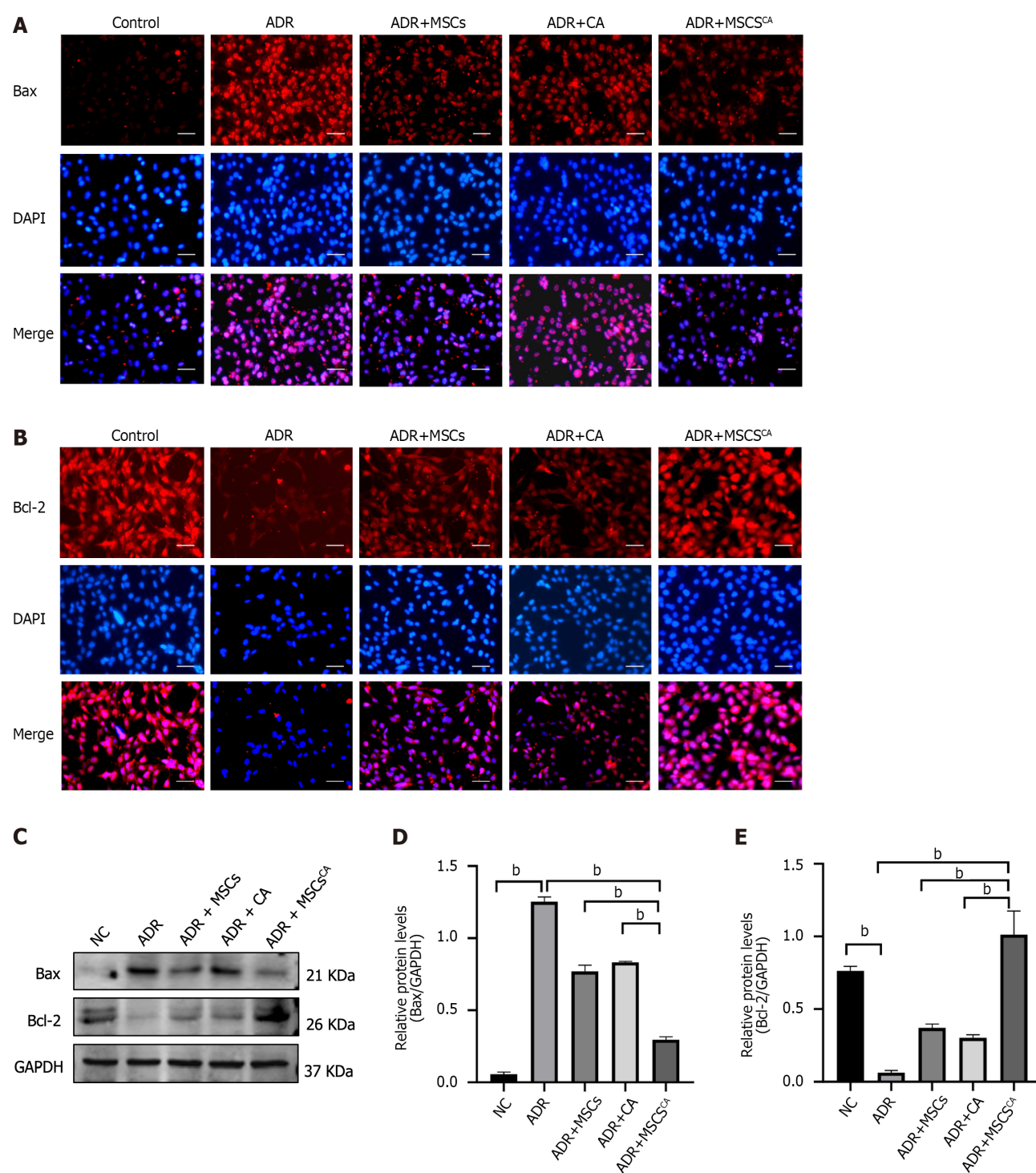
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Figure 4 Calycosin pretreatment enhances the ability of mesenchymal stem cells to ameliorate the injury of adriamycin-stimulated mouse podocyte cells *in vitro*. A: Podocin expression in each group as determined by immunofluorescence staining. Bar = 50 μ m; B: Analysis of relative (podocin/GAPDH) mRNA expression by real-time quantitative polymerase chain reaction. Data are expressed as the mean $\pm$ SD ($n = 3$). ^b $P < 0.001$; C and D: Protein expression levels of podocin detected by Western blot and normalized to control. Data are expressed as the mean $\pm$ SD ($n = 3$). ^a $P < 0.05$, ^b $P < 0.001$. NC: Normal control; ADR: Adriamycin; MSCs: Mesenchymal stem cells; CA: Calycosin; MSCs^{CA}: Mesenchymal stem cells pretreated with calycosin.

drugs that causes podocyte apoptosis due to its pharmacological action and distribution[58]. However, how to protect podocytes from ADR needs further research to find more effective targeted drugs.

Smad3 is involved in apoptosis, and podocytes are no exception[59,60]. Activation of Smad3 and its related pathway proteins induces podocyte apoptosis[14,61]. The canonical Smad pathway is a crucial regulatory route in the etiology of renal inflammation and fibrosis, according to earlier research. Major receptor-associated Smads include Smad2 and Smad3. Mad-homology 2 domain is located at the C-terminus of Smad3, which has unique phosphorylation sites and sequences triggered by transforming growth factor (TGF)- β 1. The binding of phosphorylated Smad3 to TGF- β 1 signaling receptors promotes fibrosis[62]. Our study showed that MSCs^{CA} significantly downregulated the expression of p-Smad3 in the kidneys of ADR-induced FSGS mice and ADR-induced MPC5 cells. Subsequently, we overexpressed Smad3 in MPC5 cells and confirmed that MSCs^{CA} targeted inhibition of p-Smad3 to improve podocyte apoptosis using rescue experiments. This provides a new possible mechanism and target for preventing podocyte apoptosis by MSCs^{CA}.

There were some limitations to this study. Although we have revealed that MSCs^{CA} improve podocytes apoptosis by inhibiting Smad3 signaling, this study still has certain limitations and the underlying mechanism deserves further exploration. First, how does MSCs^{CA} intervene in the Smad3 signal, directly or indirectly? We speculate that CA may activate the anti-apoptotic activity of MSCs or affect the differentiation, mobilization, and homing of BMSCs as well as the abundance of beneficial exosomes, but the main mechanism and responsible factors are still unknown. Second, it is still unclear which molecules in podocytes respond to the activity of MSCs and what are their potential relationship with Smad3. Understanding these mechanisms is conducive in expanding the application of MSCs^{CA}, and we will answer each question one by one in future research.

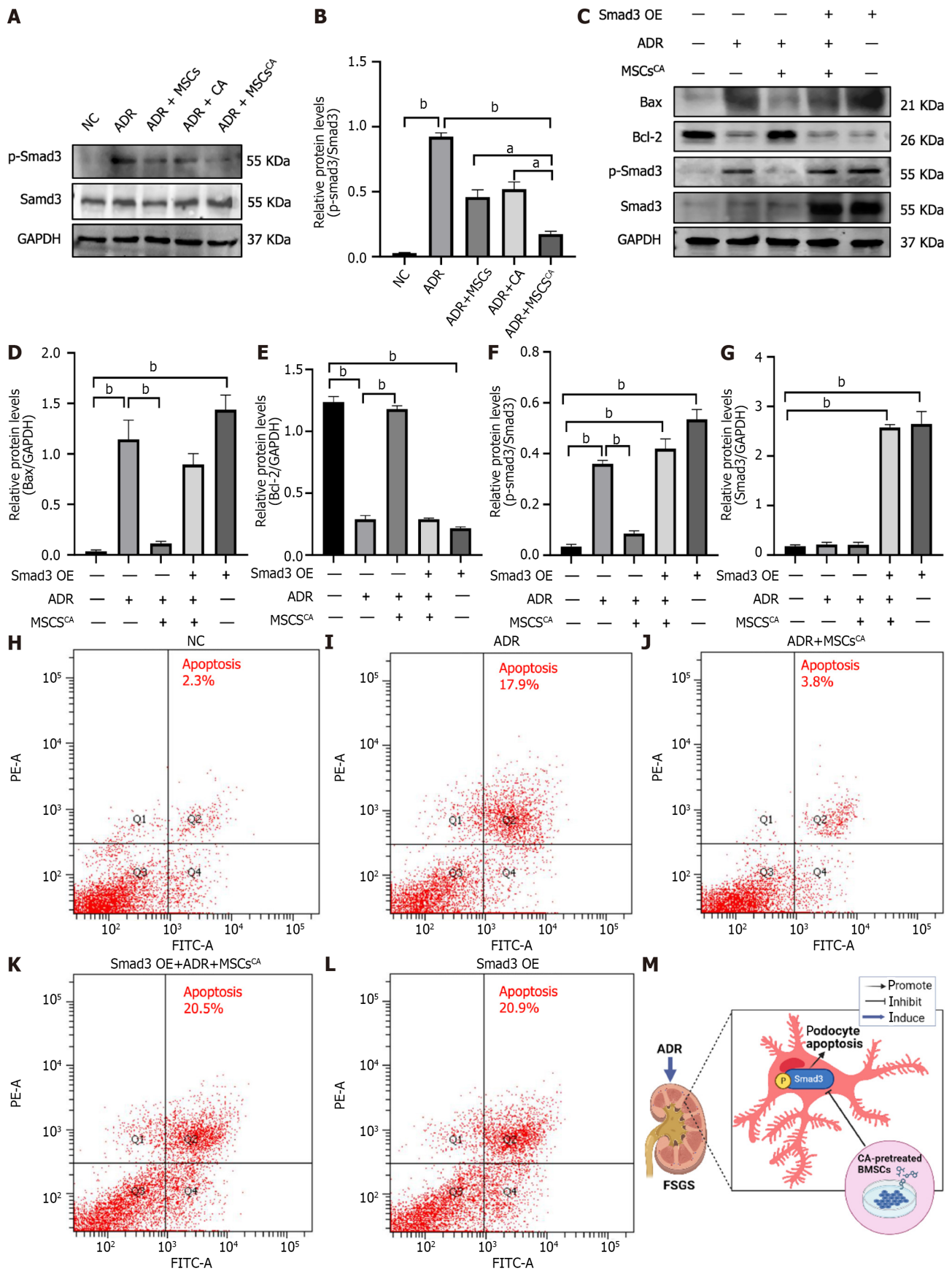


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Figure 5 Calycosin pretreatment enhances the ability of mesenchymal stem cells to inhibit apoptosis in adriamycin-stimulated mouse podocyte cells. A and B: Expression of Bax and Bcl-2 in each group as determined by immunofluorescence staining. Bar = 50 μ m; C-E: Protein expression levels of Bax and Bcl-2 detected by Western blot and normalized to control. Data are expressed as the mean $\pm$ SD ($n = 3$). ^b $P < 0.001$. NC: Normal control; ADR: Adriamycin; MSCs: Mesenchymal stem cells; CA: Calycosin; MSCs^{CA}: Mesenchymal stem cells pretreated with calycosin.

CONCLUSION

This study showed that MSCs^{CA} improve ADR-induced podocyte apoptosis by targeting Smad3 inhibition, and are superior to MSCs or CA. Thus, our study provides a new perspective on the synergistic application of MSCs and a new theory for the mechanism of improvement of podocyte apoptosis.



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Figure 6 Calycosin-pretreated mesenchymal stem cells improve adriamycin-induced podocyte apoptosis by targeting p-Smad3 expression. A and B: Protein expression levels of p-Smad3 and Smad3 in mouse podocyte cells (MPC5) detected by Western blot and normalized to control. Data are expressed as the mean $\pm$ SD ($n = 3$). ^a $P < 0.05$, ^b $P < 0.001$; C-G: Protein expression levels of Bax, Bcl-2, p-Smad3, and Smad3 in Smad3-overexpressing MPC5

cells detected by Western blot and normalized to control. Data are expressed as the mean $\pm$ SD ($n = 3$). ^b $P < 0.001$; H-L: Cell apoptosis detected by flow cytometry; M: Graphical abstract (created in BioRender.com). NC: Normal control; ADR: Adriamycin; MSCs: Mesenchymal stem cells; CA: Calycosin; MSCs^{CA}: Mesenchymal stem cells pretreated with calycosin; FSGS: Focal segmental glomerulosclerosis.

ARTICLE HIGHLIGHTS

Research background

Focal segmental glomerulosclerosis (FSGS) has become a global public health problem due to its high incidence and lack of treatment. Prevention of podocyte apoptosis is essential in the treatment of FSGS. Bone marrow-derived mesenchymal stem cells (BMSCs) have been found to protect podocytes, but have some limitations, such as low survival rate *in vivo* and poor homing function. In our previous study, calycosin (CA)-pretreated BMSCs enhanced the antifibrotic activity in kidneys compared with BMSCs. Therefore, CA-pretreated MSCs are expected to be a new method to protect podocytes in the treatment of FSGS.

Research motivation

Although MSCs have been confirmed to improve podocyte apoptosis in mice, their availability and effectiveness *in vivo* are limited. Currently, there is still a lack of effective therapeutic methods for FSGS, and their mechanism of action is not clear.

Research objectives

To evaluate the therapeutic effect of CA-pretreated BMSCs in a mouse model of adriamycin (ADR)-induced FSGS *in vivo* and MPC5 cells *in vitro*.

Research methods

MSCs^{CA} were compared with MSCs or CA to observe their inhibitory effects on podocyte apoptosis in mice with ADR-induced FSGS *in vivo* and ADR-treated MPC5 cells *in vitro*, to explore the possible mechanism by which MSCs^{CA} improves podocyte apoptosis.

Research results

In vivo results showed that MSCs^{CA} reduced podocyte apoptosis, improved podocyte injury and depletion, alleviated glomerulosclerosis and albuminuria, and downregulated p-Smad3 expression in ADR-induced FSGS mice, which were superior to MSCs and CA. Similar to *in vivo* studies, MSCs^{CA} alleviated ADR-induced apoptosis of MPC5 cells more significantly than MSCs and CA. Through rescue experiments, we found that the potential of MSCs^{CA} to protect podocytes may be realized through targeted inhibition of p-Smad3 expression.

Research conclusions

MSCs^{CA} improve ADR-induced podocyte apoptosis by targeting Smad3 inhibition, which are superior to MSCs or CA.

Research perspectives

Our findings provide a new potential strategy for the treatment of FSGS.

FOOTNOTES

Author contributions: Wang L and Fahsai K contributed equally to this article as co-corresponding authors; Wang L and Fahsai K designed the study and supervised the laboratory experiments; Hu QD conducted the experiments and drafted the manuscript; Tan RZ assisted with the experiments; Zou YX and Li JC collected the samples; Fan JM and Wang L contributed new reagents and analytic tools; and all authors read and approved the final manuscript.

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