Protective effects of MSC-conditioned medium on DFO-induced mimetic-hypoxia injury of renal tubular epithelial cells

Tianbiao Zhou (zhoutb@aliyun.com)
Shantou University https://orcid.org/0000-0001-8065-1644
Chunling Liao
Shantou University Medical College
Wenjuan Weng
Shantou University Medical College
Lujun Yang
Shantou University Medical College
Yiping Liu
Shantou University Medical College
Qian Yang
Shantou University Medical College
Guangyong Chen
Shantou University Medical College

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Abstract

Background: Acute kidney injury (AKI) is mainly characterized by inflammatory infiltration, the damage and death of renal tubular epithelial cells (RTECs), in which hypoxia plays an important role. Deferoxamine (DFO) inducing cells' mimetic-hypoxia injury is a well-accepted renal injury model in vitro. Mesenchymal stem cell - conditioned medium (MSC-CM) can reduce local inflammation and repair tissue. In this study, we explored the effect of MSC-CM on RTECs under DFO mimetic-hypoxia and its possible molecular mechanism.

Methods: NRK-52E cells with the treatment of DFO25uM for 24 hours could be well-accepted RTECs' injury model using Cell Counting Kit-8 (CCK-8) to detect cell viability and Western Blot to evaluate the expression of transforming growth factor-beta 1 (TGF-β1), α-smooth muscle actin (α-SMA), hypoxia-inducible factor-1 alpha (HIF-1α) in NRK-52E cells with the treatment of different concentrates of DFO for 24 hours. Then three groups of NRK-52E cells were used in experiments, including normal control (NC), DFO25uM, DFO25uM+MSC-CM. MSC-CM was used to culture cells for 12 hours before DFO treatment, then fresh MSC-CM and DFO 25uM cocultured cells for another 24 hours. Then the cell samples were collected.

Results: Using Western Blot and cellular immunofluorescence, we found MSC-CM could reverse the DFO-induced up-regulation of TGF-β1, α-SMA, HIF-1α and steroid receptor coactivator 1 (SRC-1). DFO 25uM coculturing NRK-52E cells for 24 hours could simulate hypoxia well; HIF-1α and SRC-1 might be the harmful factors promoting EMT in NRK-52E cells during DFO mimetic-hypoxia.

Conclusions: Our results suggest that MSC-CM have a protective effect on RTECs under DFO mimetic-hypoxia by down-regulating HIF-1α and SRC-1 which may be the harmful factors in renal injury.

1. Introduction

Acute kidney injury (AKI) is a common clinical syndrome characterized by a sudden decrease in glomerular filtration rate and complications, such as heart failure, electrolyte disorders and even multiple organ failure. It is mainly characterized by the damage and death of renal tubular epithelial cells (RTECs) [1], especially proximal renal tubule segment with high metabolic activity [2]. Once AKI occurs, the surviving RTECs regenerate and repair; normal repair can restore the integrity of renal tubules, but maladaptive or incomplete repair can result in the occurrence of epithelial-mesenchymal transition (EMT) and ECM deposition, promoting renal fibrosis and eventually leading to CKD or ESRD [3, 4]. In AKI caused by different conditions, hypoxia is a key driver contributing to the death and injury of RTECs [5]. And AKI still remains a worldwide public health problem because of its rising morbidity, significant mortality and lack of specific targeted treatment [4, 6]. Therefore, it is very urgent to explore a new therapeutic target and an effective intervention to promote the tissue repair of AKI.

Mesenchymal stem cell-conditioned medium (MSC-CM) is a medium rich in various regulatory factors secreted by MSC, like immunomodulatory factors, angiogenic factors and nutritional factors [7, 8], which

could inhibit harmful immune response, inflammation, apoptosis and EMT, thereby promoting ischemic tissue repair and regeneration [9, 10]. MSC-CM is cell-free, so it doesn't have features like self-replication, ectopic differentiation, tumorigenesis and genetic instability, but has lower immunogenicity. MSC-CM also has the potential for artificial modification, such as grading, concentration, binding with various carriers for packaging, transportation and maintaining the curative effect, etc. [11]. Therefore, MSC-CM is expected to be an acellular and safe therapeutic agent for the treatment of AKI and renal fibrosis. However, MSC-CM contains complex components, and the protective mechanism has not been thoroughly studied, so further research is needed to find specific therapeutic targets for more stable and effective treatment of AKI.

Hypoxia-inducible factor (HIF) is an important nuclear transcription factor maintaining oxygen homeostasis, which was first discovered by Semenza in 1992 [3]. It consists of regulatory α subunit and structural β subunit [12]. According to the difference of α subunit, it can be divided into three subtypes: HIF-1, HIF-2 and HIF-3 [3]. And HIF-1 is the central molecule in the oxygen sensitive mechanism [13]. In contrast to HIF-1β, the expression and activity of hypoxia-inducible factor-1 alpha (HIF-1α) are regulated by oxygen levels [14]. In normoxia, the proline residue of HIF-1α is hydroxylated by prolyl hydroxylases (PHDs) and then recognized by Von Hippel-Lindau (VHL) protein for subsequent ubiquitination and degradation. In hypoxia, the activity of PHDs is inhibited to prevent the hydroxylation and hydrolysis of HIF-1α, resulting in the accumulation of HIF-1α. HIF-1α interacts with HIF-1β to form a dimer HIF-1 and then HIF-1 binds to hypoxia response elements (HRE) in the regulatory region of the nucleus to activate downstream target genes, such as Erythropoietin (EPO), vascular endothelial growth factor (VEGF), Twist and B lymphoma Mo-MLV insertion region homolog 1 (Bmi1), eventually inducing related hypoxia responses like angiogenesis, cell proliferation and apoptosis [3, 13, 15-17]. At present, more and more studies have found that HIF-1α may be one of the key regulators of renal tubular hypoxia injury and renal fibrosis [3, 15, 17, 18].

Steroid receptor coactivator 1 (SRC-1) is the first real transcriptional coregulator [19], which was first discovered in yeast two-hybrid screening in 1995 [20]. With inherent histone acetyltransferase activity [21], SRC-1 can involve in the construction of highly specific enzyme-protein complexes by recruiting other histone acetyltransferases like CBP/p300, P/CAF, and histone methyltransferases like coactivator-associated arginine methyltransferase-1 (CARM1) and protein arginine N-methyltransferase-1 (PRMT1) [14], thus activating the transcription of target genes effectively and successfully. It has been found that SRC-1 could interact with the two transactivation domains located at the C-terminal of HIF-1α in hypoxia, enhancing the transcriptional activation of hypoxia-related genes mediated by HIF-1α [14, 21]. In addition, it's also found that SRC-1 could enhance the transactivation ability of TGF-β-driven Smad3/4-specific promoter [22, 23], thus promoting EMT and renal fibrosis [24-28]. Therefore, SRC-1 may be one of the key regulators of renal injury and fibrosis.

In this study, we examined the effect of MSC-CM on RTECs with deferoxamine (DFO)-induced mimetic-hypoxia injury and explored whether MSC-CM played a role by regulating the expression of HIF-1α and SRC-1.

2. Materials And Methods

2.1 Cell line and culture

NRK-52E, a normal rat kidney epithelial-like cell line, was obtained from Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium/low glucose (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin, at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.2 Generation of concentrated and sterilized MSC-CM:

Human umbilical cord-derived MSC-CM (huMSC-CM) was provided by the Regenerative Medicine team of the Transforming Medicine Research Center of the second affiliated Hospital of Shantou University. Human umbilical cord-derived MSCs were cultured in serum-free medium under the 3-D framework. The conditioned medium was collected and centrifuged in 1000rpm at room temperature for 5min to discard cell debris and dead cells. Then with ultrafiltration centrifuge tube (10kDa), the conditioned medium without cell debris and dead cells was centrifuged again in 2500g at 4℃ for 2 hours to be concentrated, followed by the evaluation of protein concentration using enhanced BCA protein assay kit (Biyun Sky, China, catalog number: P0010) and the sterilization by filtration using 220nm pinhole filtration membrane. Finally, sterilized MSC-CM with protein concentration of 10ug/ul was obtained and stored at -80℃.

2.3 Experimental design

The experiment was divided into two parts:

(1) Establishment of anoxic injury model of NRK-52E cells induced by DFO:

Cells were divided into different groups: (I) normal control (NC) group: cells were cultured in normal medium for 24 hours; (II) different concentrations of DFO group: cells were cultured for 24 hours in the medium respectively containing DFO5uM、10uM、25uM、50uM and 100uM. After the treatment, the cell samples were taken for Cell Counting Kit-8 (CCK-8) detection and Western Blot.

(2) The effect of MSC-CM on DFO-induced anoxic injury in NRK-52E cells and its possible mechanism:

Cells were randomly divided into 3 groups. They were treated as follows: (I) NC group: cells were cultured in normal medium for 24 hours;(II) DFO25uM group: cells were cultured in medium containing DFO25uM for 24 hours; (III) DFO25uM+MSC-CM group: cells were cultured for 12 hours in MSC-CM mixed medium which contained 40ul of concentrated MSC-CM per 1ml of complete medium, and then cultured in MSC-CM mixed medium containing DFO25uM for 24 hours. After the treatment, the cell samples were taken for morphological observation, Western Blot and immunofluorescence.

2.4 Cell Counting Kit-8 (CCK-8) assay

DFO (concentrations of 0, 5, 10, 25, 50 and 100uM) was added to act on NRK-52E for 24 h, then 10ul of CCK-8 reagent was added to medium and incubated cells for 2 hours. Finally, the absorbance value was detected at 450 nm. Cell viability was calculated as (absorbance value of experimental group–absorbance value of blank control group) / (absorbance value of NC group–absorbance value of blank control group) ×100%.

2.5 Western blot analysis

The NRK-52E cells treated with DFO or MSC-CM were lysed and proteins were extracted. The quantified proteins were then isolated by SDS-PAGE, transferred onto PVDF membrane, and sealed with TBST containing 5% skim milk powder for 1 h, followed by rinsing in TBST for 3 times, overnight incubation with corresponding primary antibodies α-smooth muscle actin (α-SMA) (Cell Signaling Technology; 19245, 1:1000), transforming growth factor-beta 1 (TGF-β1)(Abcam; ab215715, 1:1000), HIF-1α (Cell Signaling Technology; 14179, 1:1000), SRC-1(Cell Signaling Technology; 2191, 1:1000), and α-Tubulin (Cell Signaling Technology; 3873, 1:1000)] at 4°C, TBST rinsing on the next day, 60min incubation with horseradish peroxidase (HRP)-labeled secondary antibody (goat anti-rabbit, goat anti-mouse) on the shaker at room temperature, TBST rinsing, 2-min coloration with ECL chemiluminescence reagent, X-ray exposure, and photography using the BIO-RAD ChemiDoc XRS+ image acquisition system.

2.6 Immunofluorescence

NRK-52E cells were fixed with 4% paraformaldehyde (Biyun Sky, China, catalog number: P0099) for 15 min at room temperature and then washed three times with Immunol Staining Wash Buffer (Biyun Sky, China, catalog number: P0106C) for 3 min at a time. Then they were permeabilizated with Enhanced Immunostaining Permeabilization Solution (Biyun Sky, China, catalog number: P0097) at room temperature for 20 min followed by the wash with Immunostaining Detergent. Cells were then blocked in Immunol Staining Blocking Buffer (Biyun Sky, China, catalog number: P0102) for 1 hour at room temperature, followed by incubation with primary antibodies [α-SMA (Cell Signaling Technology; 19245, 1:100), TGF-β1 (Abcam; ab215715, 1:100), HIF-1 α (Cell Signaling Technology; 14179, 1:50), SRC-1(Cell Signaling Technology; 2191, 1:100)] overnight at 4 °C. After the wash with Immunol Staining Wash Buffer for three times, the cells were incubated with Alexa Fluor 488-labeled secondary antibody (Biyun Sky, China, catalog number: A0423) for 1 hour at dark and room temperature, followed by the wash with Immunol Staining Wash Buffer. Then Antifade Mounting Medium with DAPI (Biyun Sky, China, catalog number: P0131) was added to stain Nuclei and resist fluorescence quenching. After 3min, fluorescence images could be obtained using Laser Scanning Confocal Microscopy (LSM800, Zeiss Company, Germany).

2.7 Scanning electron microscope (SEM) detection

NRK-52E cells were fixed with 2.5% glutaraldehyde at 4 ℃ and at the dark for 2 hours, followed by the wash with PBS for three times. And then NRK-52E cells were dehydrated respectively with 30%, 50%, 70%, 80%, and 90% ethanol for 5 minutes and then with 100% ethanol for three times (5min each time). Dry the cells indoors naturally and then observe cells' morphology under SEM (JSM 6360LA, Nippon Electronics, Japan).

2.8 Statistical analysis

The data were analyzed with SPSS 23.0 software. The measurement data were expressed as the mean value ± standard deviation (SD). The comparison of measurement data among multiple groups used one-way ANOVA analysis. LSD was used for pairwise comparison. Differences were considered statistically significant if $P < 0.05$.

3. Results

3.1 Construction of mimetic-hypoxia injury model of NRK-52E cells induced by DFO

3.1.1 The preliminary screening concentration of DFO decided by CCK-8.

CCK-8 was used to detect the effect of different concentrations of DFO co-culturing NRK-52E cells for 24 hours on the cell survival rate (Fig. 1). The cell survival rate in NC group was assumed to be 100%, and the ones in DFO groups, consisting of 5uM, 10uM, 25uM, 50uM and 100uM, were respectively 90.68% ±4.35%, 83.76% ±7.46%, 78.95% ±8.39%, 69.74% ±6.84% and 61.70% ±6.76%. Compared with NC group, the cell survival rates in DFO groups, consisting of 10uM, 25uM, 50uM and 100uM, significantly decreased ($p < 0.05$), while the DFO group of 5uM also decreased, but had no statistically significance ($p >0.05$). In order to avoid excessive cell death, DFO 10uM and 25uM were selected for the following Western blot detection to ensure the final concentration of DFO.

3.1.2 The final concentration of DFO decided by Western Blot

Western Blot was used to examine the protein expression of HIF-1α, TGF- β1 and α-SMA of NRK-52E cells in NC group, DFO10uM group and DFO25uM group (Fig. 2). Compared with NC group, the expression of HIF-1α, TGF- β1 and α-SMA in DFO 25uM group was significantly higher ($p > 0.05$); and in DFO10uM

group, the expression of TGF- β 1 and α-SMA was also significantly increased ($p > 0.05$), but the expression of HIF-1α was increased with no statistical significance ($p > 0.05$). Thus, we finally chose DFO 25uM to co-culture NRK-52E cells for 24 hours, which could lead to the successful construction of the model of NRK-52E cells with the chemical hypoxia injury.

3.2 The effect of MSC-CM on DFO-induced mimetic-hypoxia injury of RTECs and the potential mechanisms

3.2.1 Morphological analysis

Under inverted phase contrast microscope (axio observer a1, Zeiss Company, Germany) (Fig. 3A), in NC group NRK-52E cells were flat, polygonal and closely connected, which looked like the shape of "paving stone"; in DFO25uM group NRK-52E cells shrunk, widening the intercellular space, and a small number of suspended cells appeared; in DFO25uM+MSC-CM group most of the NRK-52E cells were flattened and the intercellular space became narrower than DFO25uM group. Apart from this, under SEM (Fig. 3B), NRK-52E cells in NC group had smooth surface; NRK-52E cells in DFO25uM group had rough surface with a large number of long flagella; and NRK-52E cells in DFO25uM+MSC-CM group had less and short flagella on the surface. Thus, DFO could cause morphological damage to NRK-52E cells, and MSC-CM had a certain therapeutic effect on this injury.

3.2.2 Effect of MSC-CM on the expression of α-SMA protein in NRK-52E cells with DFO-induced mimetic-hypoxia injury.

Immunofluorescence and Western Blot were used to detect the expression of α-SMA in NRK-52E cells:

(1) Immunofluorescence (Fig. 4A): Yellow fluorescence showed the expression of α-SMA and blue fluorescence showed the nucleus. α-SMA was expressed in both cytoplasm and nucleus. Under normal circumstances, α-SMA was less expressed in cells, while yellow fluorescence of α-SMA was significantly enhanced and aggregated in DFO25uM group, and the yellow fluorescence in DFO25uM+MSC-CM group was significantly weaker than that in DFO25uM group, but still stronger than that in normal group.

(2) Western Blot (Fig. 4B, C): the expression of α-SMA in DFO25uM group was significantly more than that in NC group（$p<0.05$）, while the expression of α-SMA in DFO25uM+MSC-CM group was significantly less than that in DFO25uM group（$p<0.05$）and more than that in NC group with no statistical significance ($p>0.05$).

3.2.3 Effect of MSC-CM on the expression of TGF-β1 protein in NRK-52E cells with DFO-induced mimetic-hypoxia

injury.

Immunofluorescence and Western Blot were used to detect the expression of TGF-β1 in NRK-52E cells:

(1) Immunofluorescence (Fig. 5A): Red fluorescence showed the expression of TGF- β1, blue fluorescence showed the nucleus, and TGF- β1 was mainly located in the cytoplasm. Under normal conditions, TGF- β1 was less expressed in cells, while red fluorescence was significantly enhanced and aggregated in DFO25uM group, and red fluorescence in DFO25uM+MSC-CM group was significantly weaker than that in DFO25uM group, but still stronger than that in NC group.

(2) Western Blot (Fig. 5B, C): The expression of TGF- β1 in DFO25uM group was significantly more than that in NC group（$p<0.05$）, while the expression of TGF- β1 in DFO25uM + MSC-CM group was significantly less than that in DFO25uM group（$p<0.05$）and more than that in NC group, but there was no statistical significance（$p>0.05$）.

3.2.4 Effect of MSC-CM on the expression of HIF-1α protein in NRK-52E cells with DFO-induced mimetic-hypoxia injury.

Immunofluorescence and Western Blot were used to detect the expression of HIF-1α in NRK-52E cells:

(1) Immunofluorescence (Fig. 6A): orange fluorescence showed the expression of HIF-1 α, blue fluorescence showed the nucleus, and HIF-1α was expressed in both cytoplasm and nucleus. Under normal conditions, HIF-1α was less expressed in cells, while orange fluorescence was significantly enhanced and aggregated in DFO25uM group. And the orange fluorescence in DFO25uM+MSC-CM group was significantly weaker than that in DFO25uM group, but still stronger than that in NC group.

(2) Western Blot (Fig. 6B, C): the expression of HIF-1α in DFO25uM group was significantly more than that in NC group（$p<0.05$）, while the expression of HIF-1α in DFO25uM+MSC-CM group was significantly less than that in DFO25uM group（$p<0.05$） and more than that in NC group, but there was no statistical significance（$p>0.05$）.

3.2.4 Effect of MSC-CM on the expression of SRC-1protein in NRK-52E cells with DFO-induced mimetic-hypoxia injury.

Immunofluorescence and Western Blot were used to detect the expression of SRC-1 in NRK-52E cells:

(1) Immunofluorescence (Fig. 7A): Green fluorescence showed the expression of SRC-1, blue fluorescence showed nucleus, and SRC-1 could be expressed in both cytoplasm and nucleus. Under normal circumstances, SRC-1 was less expressed in cells, while green fluorescence was significantly enhanced

and aggregated in DFO25uM group. And green fluorescence in DFO25uM+MSC-CM group was significantly weaker than that in DFO25uM group, but still stronger than that in NC group.

(2) Western Blot (Fig. 7B, C): The expression of SRC-1 in DFO25uM group was significantly more than that in NC group（$p<0.05$）, while the expression of SRC-1 in DFO25uM+MSC-CM group was significantly less than that in DFO25uM group（$p<0.05$） and significantly more than that in NC group（$p<0.05$）.

4. Discussion

Hypoxia is a condition of insufficient oxygen supply to cells or organisms [18], which plays an important role not only in the occurrence and development of AKI, but also in the development of CKD to ESKD [29]. In other words, hypoxia involved in the apoptosis and EMT of RTECs in AKI. Because of the lack of a hypoxia incubator to maintain low oxygen during cell studies, we chose DFO as the chemical compound to induce mimetic-hypoxia in this study [30-32]. DFO could stabilize HIF-1α under normoxia by chelating iron (Fe^{3+}) to inhibit the activity of PHDs [33]. DFO could cause iron deficiency in cells to affect the expression of cell cycle-related factors like cyclin D1, inhibiting cell proliferation and reducing cell survival rate [34, 35]. These theories could explain why, in our study, the expression of HIF-1α increased and cell viability decreased in the groups treated with DFO. In addition, TGF-β is a key fibrogenic cytokine that plays a critical role in the expression of α-SMA and the induction of kidney fibrosis; α-SMA is the hallmark of myofibroblasts, which are generally considered to be the key effector cells in the development of fibrosis [36]. Our results showed that the mimetic-hypoxia by DFO could increase the expression of α-SMA and TGF-β1. Therefore, according to the results, we found that DFO 25uM culturing NRK-52E for 24 hours could achieve some similar effects with physical hypoxia, which induced the accumulation of HIF-1α protein and the change of the expression of hypoxia-associated genes involved in EMT and ECM generation; and it could result in the relatively high cell viability which facilitated the collection of following experimental samples. DFO, as a hypoxia mimetic, has been applied in different cell lines including cardiomyocytes [37], rat salivary Pa-4 epithelial cells [38], human fetal mesencephalic NPCs (hmNPCs) [39], osteoblast [40], adipocytes [41], acute myeloid leukemic (AML) cells [42], dental pulp cells (DPC) [34]; but DFO is rarely found to be applied in RTECs including HK-2 cells and NRK-52E cells, therefore resulting in the lack of data about concentration and cultivating time of DFO to induce mimetic-hypoxia of NRK-52E cells. Taken together, our results suggest for the first time that DFO 25uM culturing NRK-52E for 24 hours can well induce mimetic-hypoxia of NRK-52E cells.

MSC-CM is rich in various regulatory factors secreted by MSC, such as prostaglandin E2, interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), hepatocyte growth factor (HGF), osteopontin and macrophage colony stimulating factor 1, etc., regulating immunity [8, 43, 44] and inhibiting inflammation, apoptosis, EMT and so on [10]. It has been found that MSC-CM has a certain therapeutic effect on renal injury induced by different conditions. In cisplatin-induced mouse AKI model, Simovic Markovic et al. found that intraperitoneal application of MSC-CM could decrease Cr and BUN, attenuate the renal pathological injury [45]. In unilateral ureteral obstruction (UUO) model of rat, Da Silva et al. found that intraperitoneal injection of MSC-CM could improve renal injury and fibrosis [10]. In rats' experimental antiglomerular

basement membrane glomerulonephritis (anti-GBM GN) induced by nephrotoxic serum nephritis, Iseri et al. [46] found that MSC-CM could improve proteinuria and renal dysfunction. In the mouse model of diabetic nephropathy (DN) induced by high-fat diet (HFD) or streptozotocin (STZ), Nagaishi et al. found that MSC-CM could reduce the exacerbation of albuminuria and renal histopathological damage [8]. In a rat model with CKD induced by 5/6 nephrectomy and following L-NG-Nitroarginine (L-NNA) as well as 6% NaCl diet, Van Koppen et al. found that human embryonic MSC- CM could reduce glomerulosclerosis and tubular damage to maintain a higher glomerular filtration rate (GFR) [47]. In our study, we found that MSC-CM could improve the morphological damage of NRK-52E cells and significantly inhibit the up-regulation of TGF-β1 and α-SMA under the DFO mimetic-hypoxia（$p<0.05$）. Here, we found for the first time that MSC-CM could have a therapeutic effect on NRK-52E cells with DFO mimetic-hypoxia injury, and we also provided a convenient and useful vitro model for further study of the molecular mechanism of MSC-CM protecting kidney.

To the best of our knowledge, the mechanisms about the protective effect of MSC-CM on kidney have been studied but not been explored thoroughly. Simovic Markovic et al. [45] found that in cisplatin-induced mouse AKI model, MSC-CM could attenuate renal injury by suppressing infiltration and activation of immune cells in inducible nitric oxide synthase (iNOS) -dependent manner. Zheng et al. [44] found that in UUO model of mouse, MSC-CM could attenuate renal injury by decreasing renal expression of TNF-α, IL-1β, IL-6, ICAM-1 as well as monocyte chemoattractant protein-1 (MCP-1) and reducing intrarenal infiltration of monocytes/macrophages as well as mature B lymphocyte. Da Silva et al. [10] found that in UUO model of rat, MSC-CM could improve renal injury and fibrosis by reducing the expression of collagen1-α-1, α-SMA, TNF-α, caspase3 and proliferating cell nuclear antigen (PCNA), which involved in inflammation, cell apoptosis, cell proliferation and EMT. Geng et al. [36] found that in human proximal tubular cells (HK2 cells) induced by TNF-α, MSC-CM could have the anti-inflammatory effects by decreasing the expression of monocyte chemoattractant protein-1(MCP-1), interleukin (IL)-8 and IL-6; and in HK2 cells induced by TGF- β, MSC-CM could block or reverse the EMT process by decreasing the expression of α-SMA, fibronectin (FN) as well as collagen IV and up-regulating the expression of E-cadherin. Iseri et al. [46] found that MSC-CM could repair anti-GBM GN in rats by increasing the levels of serum MCP-1 and promoting subsequent switch of macrophages toward the M2 phenotype. Nagaishi et al. [8] found that MSC-CM could improve DN in HFD- and STZ-diabetic mice by decreasing the infiltration of bone marrow-derived macrophages as well as the expression of intracellular adhesion molecule-1 (ICAM-1), TNF-α, TGF-β in tubular epithelial cells (TECs), and by reversing the down-regulation of zona occludens protein-1 (ZO-1) in TECs to help maintain the barrier function of epithelial cells in the kidney. In order to explore other molecular mechanisms about MSC-CM protecting kidney, we carried out experiments in which NRK-52E cells with DFO mimetic-hypoxia injury were treated with MSC-CM.

HIF-1α is not only an important regulator of hypoxic response, but also one of the key factors in the process of AKI and renal fibrosis. But it's still controversial whether HIF-1α promotes or inhibits renal injury and renal fibrosis. On one hand, some studies indicated that HIF-1 might improve cells' tolerance to conditions of hypoxia, thus reducing the apoptosis of renal cell, promoting the repair of acute renal tubular necrosis and finally protecting kidney from AKI [5, 18]. For example, Yang et al. [48] found that in

cisplatin- induced AKI model of mice, HK2 cells and mouse proximal tubular cells (mPTCs), HIF-1α, activated by FG-4592 as a PHD inhibitor before modeling, could significantly inhibit the up-regulation of BUN, Cr, cystatin C, Kim-1 as well as NGAL, inhibit cell apoptosis and improve cisplatin-induced renal injury. Rajendran et al. [49] found that in IR-induced AKI model of mouse, HIF-1 activated by inhibiting the PHD2 in RTECs could improve renal inflammation as well as renal failure and block the transition from AKI to CKD. On the other hand, there are also some evidences indicating that in the context of hypoxia, HIF-1α could induce EMT of RTECs and ECM deposition to promote the occurrence of renal fibrosis [3, 15]. For example, Higgins et al. [50] found that in the models of mice with UUO-induced renal injury and proximal RTECs with hypoxia-induced injury in vitro, HIF-1α could promote the EMT of RTECs while genetic ablation of HIF-1α in RTECs could inhibit the development of renal tubulointerstitial fibrosis, which might be related to the decrease of interstitial collagen deposition, inflammatory cell infiltration and fibroblast-specific protein-1-expressing (FSP-1-expressing) interstitial cells. Luo et al. [51] found that in HK2 cells with hypoxia/reoxygenation (H/R)-induced injury, the activation of HIF-1α significantly could increase the expression of α-SMA, decrease the expression of E-cadherin and promote EMT. In order to further explore the role of HIF-1α in AKI and renal fibrosis, we carried out the related experiment. Our study demonstrated that HIF-1α could upregulate TGF-β and α-SMA expression during DFO mimetic-hypoxia in NRK-52E cells, which might promote the EMT process; and our results suggested for the first time that MSC-CM might protect RTECs from EMT by inhibiting the expression of HIF-1α. And the above-mentioned inconsistent results abouts the role of HIF-1α in AKI and renal fibrosis may be caused by different experimental conditions, diverse animal or cell models, different damage intensity and duration, different methods of manipulating HIF-1 activity and so on. It is worth noting that these harmful or protective mediators are not always easily distinguished. The overall effect depends on the intensity and duration of their expression. Therefore, more studies are in need to explore the role of HIF-1 in AKI and renal fibrosis.

SRC-1 as a nuclear hormone receptor coactivator, could be involved in the hypoxia response of cells by interacting with HIF-1α to enhance HIF-1α-dependent gene activation [14, 21]. In addition, SRC-1 could also interact with Smad3 to finally enhance the TGF-β-induced and Smad-mediated transcription [22], finally enhancing EMT. These might explain the increase of SRC-1 in NRK-52E cells during DFO mimetic-hypoxia. And in terms of the role of SRC-1 in the occurrence and development of diseases, more studies focused on cancers like breast cancer and nasopharyngeal carcinoma [52-55], but few studies have explored the role of SRC-1 in AKI. To our best of knowledge, our study demonstrated for the first time that SRC-1 might promote EMT in NRK-52E cells during DFO mimetic-hypoxia, which might be a novel and significant target for the treatment of AKI and its progression to CKD. In addition, we also found for the first time that MSC-CM might protect NRK-52E cells from EMT by inhibiting the expression of SRC-1.

5. Conclusions

In conclusion, our study demonstrated for the first time that DFO 25uM coculturing NRK-52E cells for 24 hours could simulate hypoxia well; HIF-1α and SRC-1 might be the harmful factors promoting EMT in NRK-52E cells during DFO mimetic-hypoxia; and MSC-CM might have a protective effect on NRK-52E cells during DFO mimetic-hypoxia by inhibiting the expression of HIF-1α and SRC-1 to inhibit EMT. Therefore,

we speculate that HIF-1α and SRC-1 might be the target for the treatment of AKI and MSC-CM might have a protective effect on AKI by regulating HIF-1α and SRC-1. And further studies, including animal experiments, are necessary to explore the renoprotective mechanisms of MSC-CM in AKI.

Abbreviations

AKI: Acute kidney injury; RTECs: renal tubular epithelial cells; DFO: deferoxamine; MSC-CM: mesenchymal stem cell - conditioned medium; CCK-8: cell counting Kit-8; TGF-β1: transforming growth factor-beta 1; α-SMA: α-smooth muscle actin; HIF-1α: hypoxia-inducible factor-1 alpha; NC: normal control; SRC-1: steroid receptor coactivator 1; EMT: epithelial-mesenchymal transition; HIF: hypoxia-inducible factor; PHDs: prolyl hydroxylases; VHL: Von Hippel-Lindau; HRE: hypoxia response elements; EPO: erythropoietin; VEGF: vascular endothelial growth factor; Bmi1: B lymphoma Mo-MLV insertion region homolog 1; CARM1: coactivator-associated arginine methyltransferase-1; PRMT1: protein arginine N-methyltransferase-1; DMEM: Dulbecco's modified Eagle's medium/low glucose; FBS: supplemented with 10% fetal bovine serum; huMSC-CM: human umbilical cord-derived MSC-CM; hmNPCs: human fetal mesencephalic NPCs; AML: acute myeloid leukemic; IL-10: interleukin-10; TNF-α: tumor necrosis factor-α; HGF: hepatocyte growth factor; UUO: unilateral ureteral obstruction; DN: diabetic nephropathy; HFD: high-fat diet; STZ: streptozotocin; GFR: glomerular filtration rate; iNOS: inducible nitric oxide synthase; PCNA: proliferating cell nuclear antigen; FN: fibronectin; ZO-1: zona occludens protein-1.

Declarations

Funding:

None.

Authors' contribution

TBZ was in charge of conceived and designed the study. CLL, WJW and YPL were responsible for conducting the experiments, and performing the statistical analysis and manuscript preparation. LJY provided the MSC-CM. QY and GYC were responsible for checking the data. CLL were responsible for drafting the manuscript, and TBZ modified and polished the article. All the authors approved the final version.

Data Availability

All data generated of this study are included in the published article. The data used in this study are available on request from the corresponding author.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

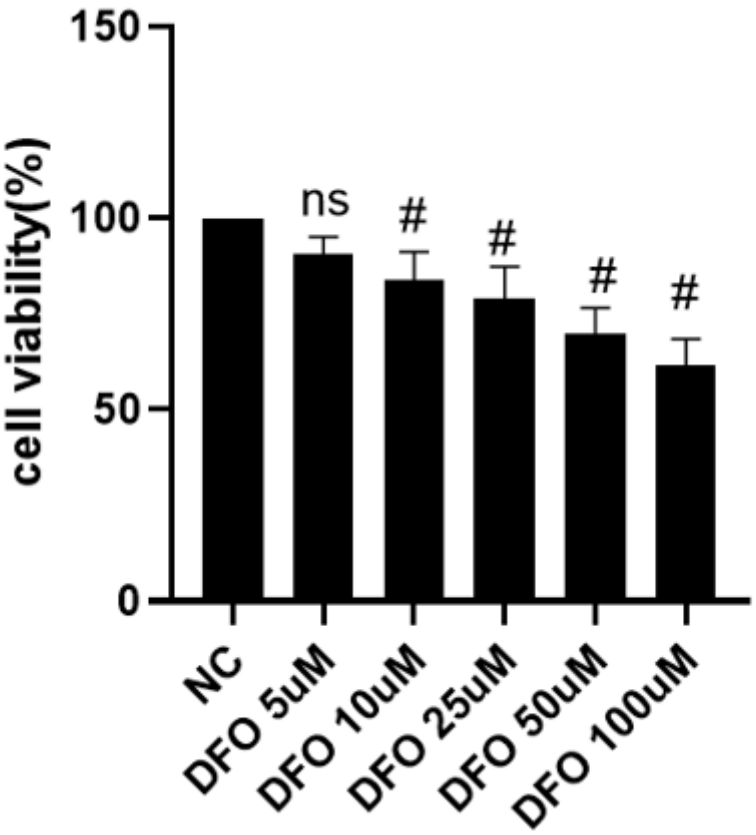

Figure 1

The effect of different concentrations of DFO co-culturing NRK-52E cells for 24 hours on the cell survival rate. Compared with NC group: #, p <0.05; ns, not significantly

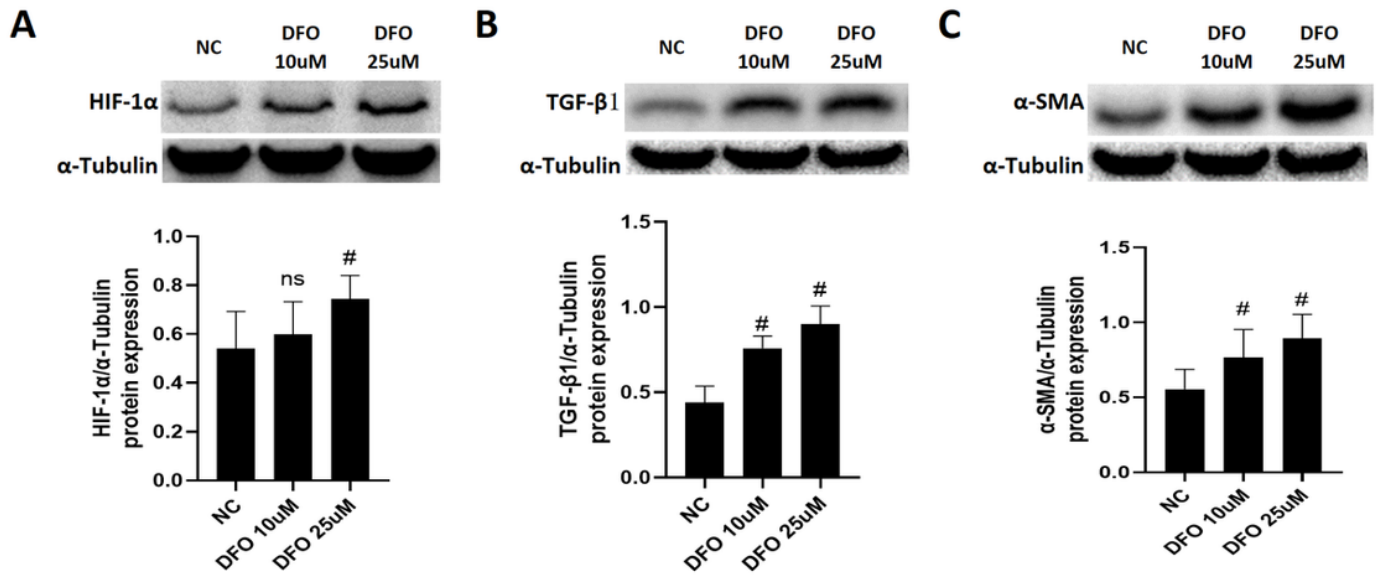

Figure 2

The effect of different concentrations of DFO co-culturing NRK-52E cells for 24 hours on the expression of HIF-1α(A), TGF- β1(B) and α-SMA(C). Compared with NC group: #, p <0.05; ns, no significance.

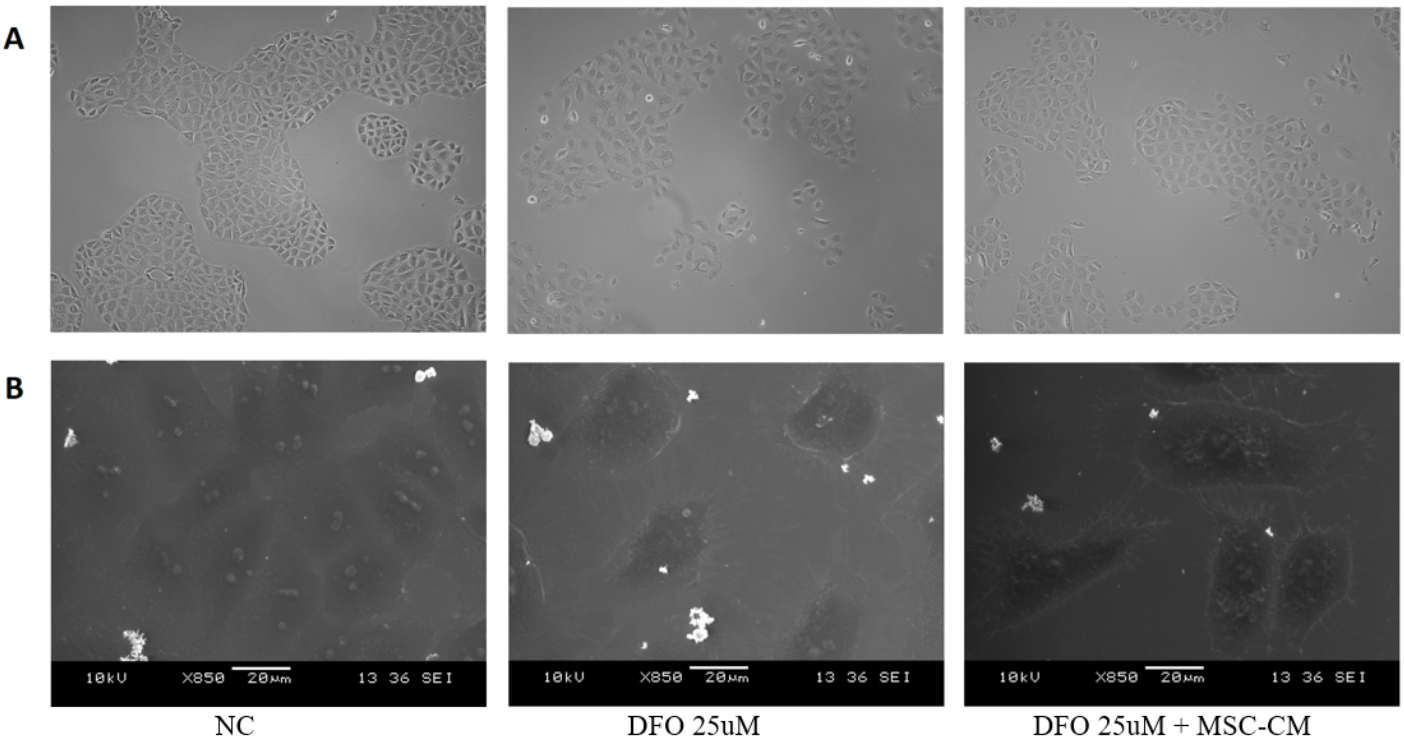

Figure 3

Morphological analysis of cells in NC group, DFO 25uM group and DFO 25uM + MSC-CM group. A: inverted phase contrast microscope (10X); B: Scanning electron microscope (850X)

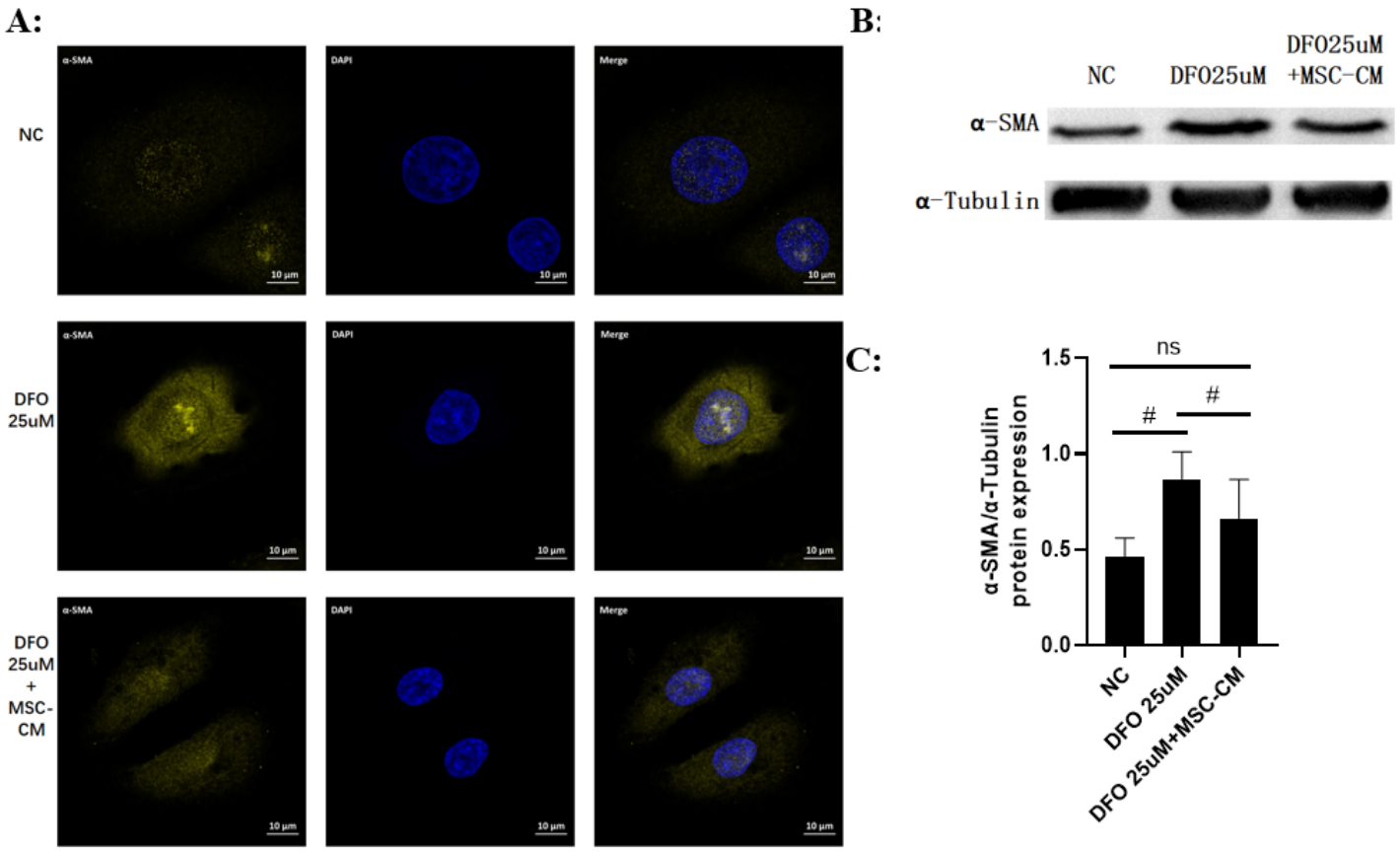

Figure 4

The protein expression of α-SMA of NRK-52E cells in NC group, DFO 25uM group and DFO 25uM + MSC-CM group. A: Immunofluorescence; B, C: Western blot. #: $p < 0.05$; ns: no significance ($p > 0.05$).

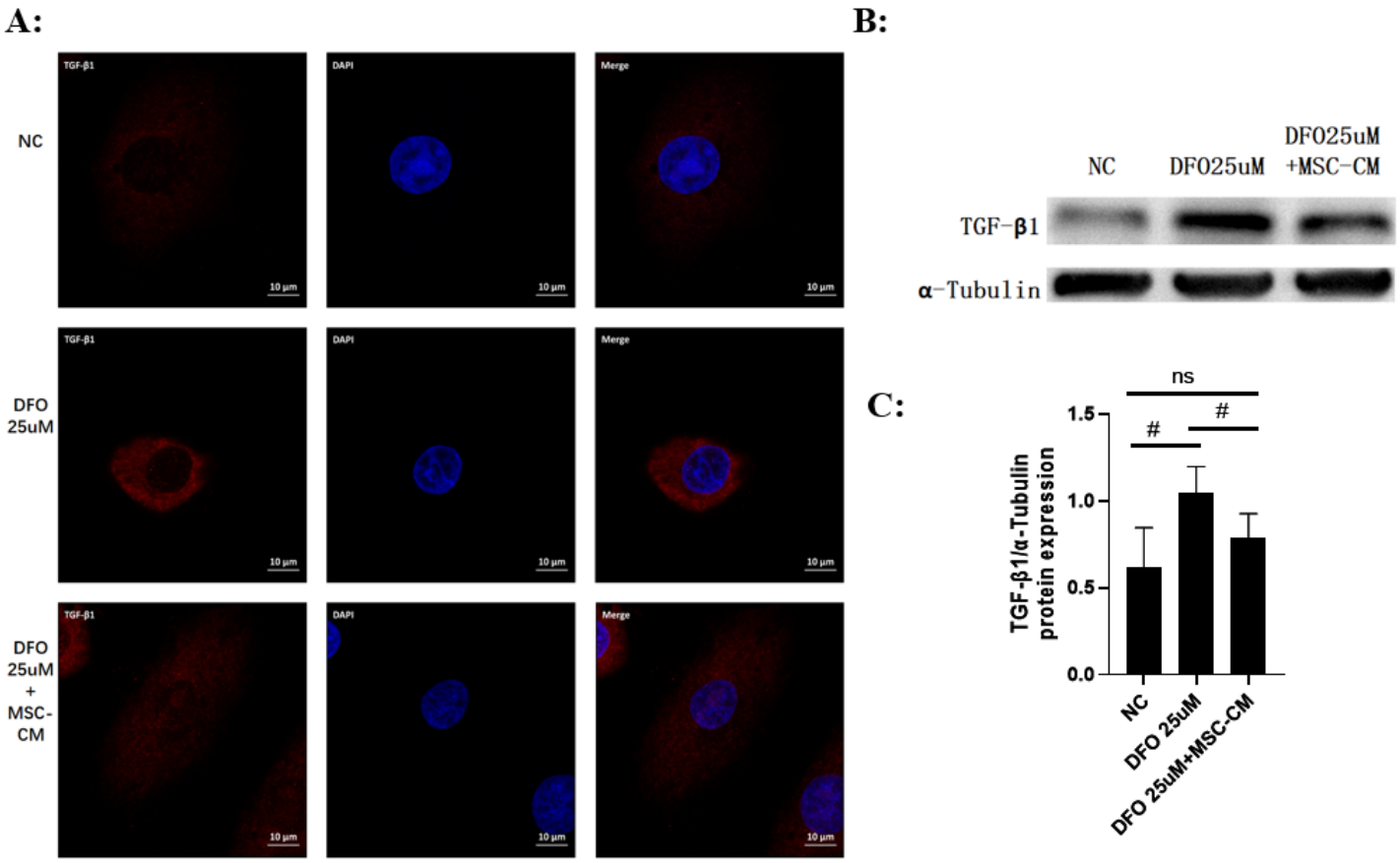

Figure 5

The protein expression of TGF- β1 of NRK-52E cells in NC group, DFO 25uM group and DFO 25uM + MSC-CM group. A: Immunofluorescence; B, C: Western blot. #: p <0.05; ns: no significance (p >0.05).

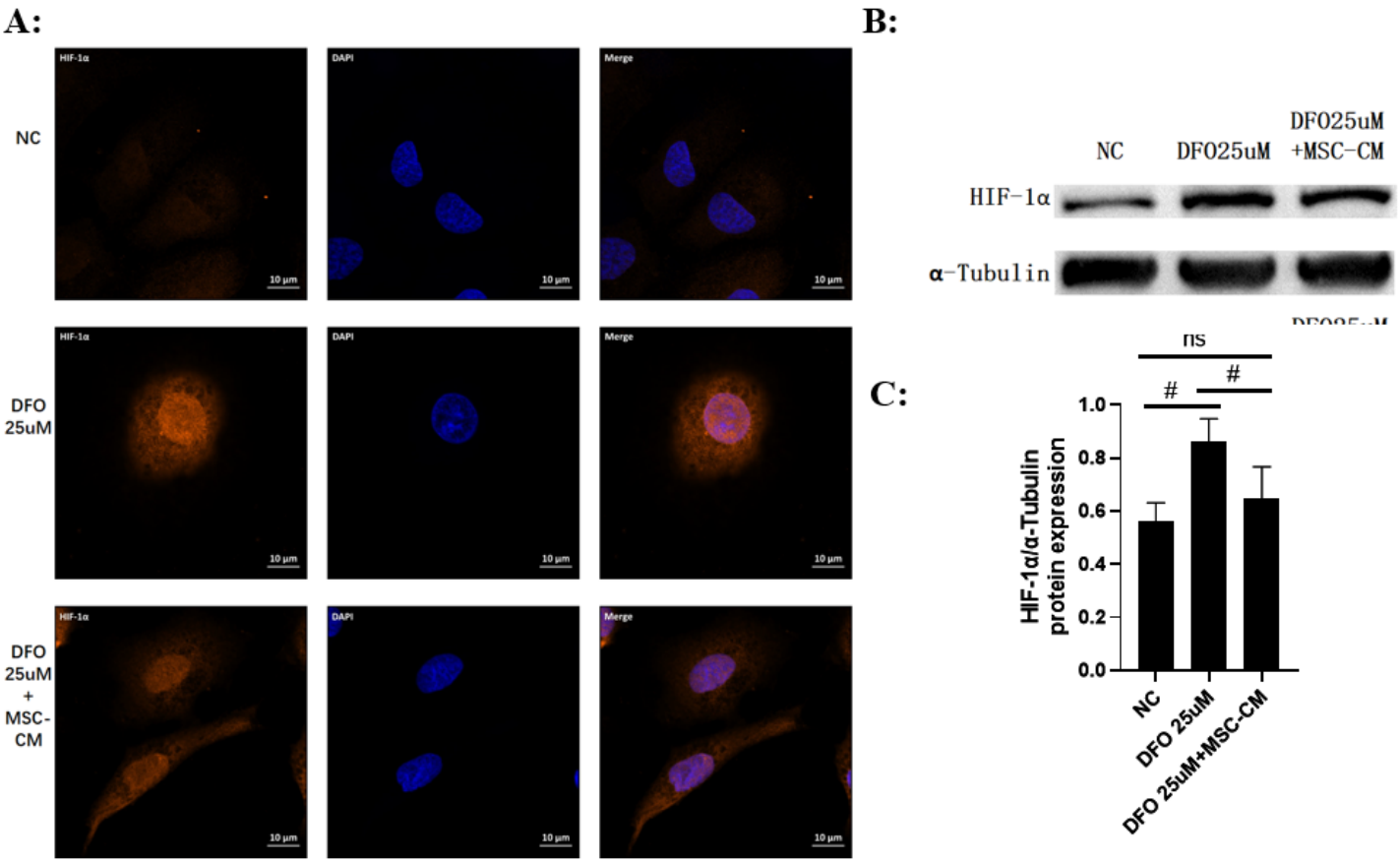

Figure 6

The protein expression of HIF-1α of NRK-52E cells in NC group, DFO 25uM group and DFO 25uM + MSC-CM group. A: Immunofluorescence; B, C: Western blot. #: $p < 0.05$; ns: no significance ($p > 0.05$).

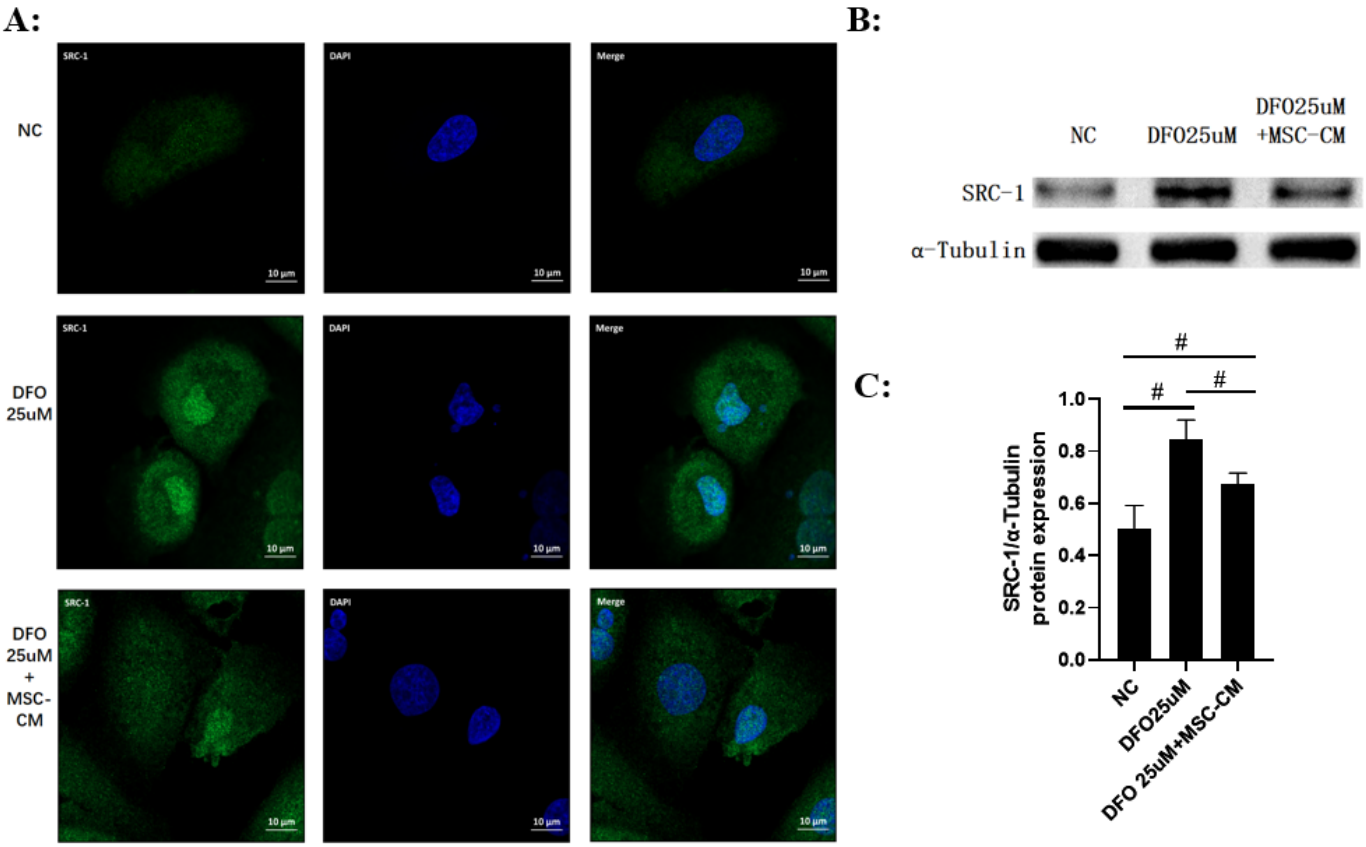

Figure 7

The protein expression of SRC-1 of NRK-52E cells in NC group, DFO 25uM group and DFO 25uM + MSC-CM group. A: Immunofluorescence; B, C: Western blot. #: $p < 0.05$.