Protective effect of *GDNF*-engineered amniotic fluid-derived stem cells on the renal ischaemia reperfusion injury in vitro

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Abstract

Objectives: Amniotic fluid-derived stem cells (AFSCs) possessing multilineage differentiation potential are proposed as a novel and accessible source for cell-based therapy and tissue regeneration. Glial-derived neurotrophic factor (GDNF) has been hypothesized to promote the therapeutic effect of AFSCs on markedly ameliorating renal dysfunction. The aim of this study was to investigate whether AFSCs equipped with GDNF (GDNF-AFSCs) had capabilities of attenuating mouse renal tubular epithelial cells (mRTECs) apoptosis and evaluate its potential mechanisms.

Materials and methods: A hypoxia-reoxygenation (H/R) model of the mRTECs was established. Injured mRTECs were co-cultured with GDNF-AFSCs in a transwell system. The mRNA expressions of hepatocytes growth factor (HGF) and fibroblast growth factor (bFGF) were detected by qRT-PCR. Changes of intracellular reactive oxygen species (ROS) and the level of superoxide dismutase (SOD) and malondialdehyde (MDA) were examined. The expressions of nitrotyrosine, Gp91-phox, Bax, and Bcl-2 were determined by Western blotting. Cell apoptosis was assayed by flow cytometry, and caspase-3 activity was monitored by caspase-3 activity assay kit.

Results: Our study revealed that expression of growth factors was increased and oxidative stress was dramatically counteracted in GDNF-AFSCs-treated group. Furthermore, apoptosis induced by H/R injury was inhibited in mRTECs by GDNF-AFSCs.

Conclusions: These data indicated that GDNF-AFSCs are beneficial to repairing damaged mRTECs significantly in vitro, which suggests GDNF-AFSCs provide new hopes of innovative interventions in different kidney disease.

1 | INTRODUCTION

Acute kidney injury (AKI) is a critical clinical problem that contributes to a high level of mortality, chronic kidney disease and end-stage renal disease (ESRD).¹ Although renal replacement therapy technology and critical care treatment level increase significantly, there is currently no optimal treatment for patients to prevent disease progression.

Renal ischaemia reperfusion injury (RIRI), regarded as a risk factor for transplanted kidneys,² is a hallmark and significant causative factor of AKI.^{2,3} During the process of RIRI, a sequence of events occur, such as the renal tubular epithelial cells apoptosis and necrosis, the accumulation of reactive oxygen metabolites,⁴ calcium overload,⁵ as well as the increasing number of NO, inflammatory mediators and energy metabolism disorder and so on. It will result in kidney function disorder thus causing metabolic abnormalities. Given the shortage of donor organs, usage of marginal donor kidneys for transplantation as well

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as the high incidence of AKI, early diagnosis and the development of effective therapeutic strategies are essential.

With the rise and development of tissue engineering, stem cells have become the focus of life science. According to the different developmental stages, it can be divided into embryonic stem cells (ESCs) and adult stem cells (ASCs). Amniotic fluid-derived stem cells (AFSCs) between adult stem cells and ESCs, characterized by expression of the surface antigen c-kit (CD117),⁶ are able to give rise to lineages representing the 3 germ layers, such as osteoblasts, myocytes, cardiomyocytes, chondrocytes and adipocytes.^{7–11} AFSCs have been considered as one of the most likely sources of endogenous tissue repair in the light of self-renewal ability, high proliferative potential, lack of immunogenicity, low risk of tumorigenicity, safety considerations and absence of ethical concerns.^{12,13} Their efficacy in the repair of kidney injury has been described in several studies.^{14,15} In addition to differentiating into kidney cells,¹⁶ AFSCs can improve renal function by limiting tubular damage.¹⁷ Moreover, they are beneficial to reducing inflammation reaction and inhibiting the renal fibrosis, so as to slow down the progress of chronic renal failure when injected in a murine model of unilateral ureteral obstruction.¹⁸ Similar result was verified in a pre-clinical porcine model of renal transplantation.¹⁹ Surveys such as that conducted by Perin L have shown that AFSCs play a central role in acute tubular necrosis.²⁰ However, the underlying mechanisms which affect tissue repair and regeneration remain to be understood. What is widely recognized is mainly by migrating to the renal tissue and differentiating into the renal cells.¹⁶ While this latter discovery provided new insights into the pivotal role played by AFSCs secretome.²¹ Their secretion of cytokines participate in the repair of renal tubular structure and function to ameliorate the local microenvironment, which makes them strong candidates for cell therapy in the long run.

Glial cell line-derived neurotrophic factor (GDNF) is one of the dopaminergic neuron nutritional factor, which was first purified from B49 glial cells and exists throughout the central nervous system and part of the peripheral system. Accumulating evidences have indicated that GDNF exerts potent neuroprotective effects in different experimental models of brain injury²² by way of increasing the viability of ischaemic tissues. More paramount importance is to reduce the overall intensity of inflammation cytokines and oxidative stress, and completely block the generation of NO.^{23,24} Recent advances have boosted efforts to explore the potential of GDNF to promote the proliferation and differentiation of stem cells.²⁵ In addition, it can mediate AFSCs through stimulating the production or secretion of other beneficial factors.^{26,27} However, little is known about the detailed mechanisms underlying this process.

Therefore, this study aimed to examine whether GDNF-engineered AFSCs can enhance anti-apoptosis activity and promote cell repair in RIRI.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All methods were performed in accordance with the relevant guidelines and regulations. hAFSC samples were collected with the written

consent of subjects, and the protocol was obtained after receiving approval from the Institutional Review Board of the Affiliated Hospital of Xuzhou Medical College. The approval number is xyfylw2013032. All experimental procedures using human Amniotic Fluid Cells samples in this study were reviewed and approved by the ethics committee.

2.2 | Cell culture

Stocks of AFSCs were obtained from 15 to 20 weeks of gestation women for prenatal diagnosis in the maternal and child health hospital in XuZhou. AFSCs were resuscitated and cultured in a-modified minimum essential medium (Hyclone, Logan, NY, USA) supplemented with 20% foetal bovine serum (FBS; Hyclone, Logan, UT, USA), 4 ng/mL basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ, USA) and 1% mycillin (Beyotime, Shanghai, China). Mouse renal tubular epithelial cells (mRTECs) obtained from the Chinese Academy of Sciences, Beijing, were cultured in Dulbecco's modified Eagle's medium (DMEM)/high glucose containing 10% FBS (Gibco BRL, Grand Island, NY). All cells were grown at 37°C in a humidified incubator with 5% CO₂ and the medium was replaced every 2 or 3 days throughout the entire culture period.²⁵

2.3 | Lentivirus vector production

A green fluorescent protein (GFP) label for a lentivirus vector plasmid system carrying the GDNF gene was constructed by Shanghai Jikai Gene Technology Co., Ltd.

2.4 | Stable expression of GDNF in AFSCs

Amniotic fluid-derived stem cells were transfected with adenovirus vectors at an appropriate multiplicity of infection (MOI) = 20. Briefly, AFSCs were seeded at a density that allowed them to grow to 70%–80% confluence on the day of transduction. Cells were transfected for 8–12 hours via exposure to virus-containing supernatants in the presence of 8 µg/mL polybrene. Vector-containing medium was replaced with fresh medium, and cells were incubated at 37°C in a CO₂ incubator. GFP expression was observed by performing fluorescence microscopy at 1, 3 and 5 days after lentiviral vector transfection.

2.5 | Cell H/R

The H/R model was designed to mimic renal cell I/R injury in vitro. Before the procedure, mRTECs at 4.0×10^5 cells/well were cultured in 6-well plates and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 48 hours. At 80% confluence, the cells were washed with phosphate-buffered saline (PBS) and incubated in paraffin at 37°C, 5% CO₂ for 90 minutes.²⁸ Then we removed the paraffin, used PBS to eliminate non-attached cells and added the fresh medium to the cells.

2.6 | In vitro co-culture

After establishing the H/R model, a co-culture system was built. In this regard, mRTECs were grown in lower chamber of transwell dishes in

DMEM/high glucose medium containing 10% FBS. Simultaneously, the GDNF-AFSCs and AFSCs were cultured in the upper chambers. GDNF-AFSCs or AFSCs were co-cultivated with mRTECs in a transwell system with a 0.4 mm pore size after being treated with paraffin for 90 minutes.²⁸

2.7 | Real-time PCR analysis

Real-time PCR analysis was used to measure the paracrine effects of GDNF-AFSCs on expression of growth factors in the paraffin-treated mRTECs. Overall, total RNA from day 3 of paraffin-treated mRTECs co-cultured with GDNF-AFSCs and AFSCs were extracted with RnaEx and treated with DNaseI (Shanghai Generay Biotech Co., Ltd., Shanghai, China). Subsequently, cDNA was obtained via reverse transcription of total RNA with random hexamers and SuperScript III reverse transcriptase (Tiagen Biotech Co., Ltd., Beijing, China). Quantitative RT-PCR was performed in triplicate for each sample and each gene using a 7500 Real-Time PCR System and Power SYBR Green PCR Master Mix. The following primers were used: forward primer for HGF, TCCAGAGGTACGCTACGAAGTC; reverse primer for HGF, CGGTGTGGTGTCTGCTGATC; forward primer for bFGF, AGATTAGCGGACGCGGTG; reverse primer for bFGF, GGTTCACGGATCCCTGTCT; forward primer for β -actin, TTCTACAATGAGCTGCGTGTGG, reverse primer for β -actin, GTGTTGAAGTCTCAAACATGAT. The PCR reaction was performed as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds and 60°C for 30 seconds. Each gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

2.8 | Detection of intracellular reactive oxygen species (ROS) production and biomarkers of oxidative stress

The intracellular accumulation of ROS was determined by fluorescence microscopy using 2'-7'-Dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, mRTECs planted in 6-well plates were subjected to H/R protocol. After co-culturing with GDNF-AFSCs and AFSCs for 3 days, mRTECs were washed twice with PBS and incubated in the presence of 10 mol/L DCFH-DA in serum-free medium at 37°C for 30 minutes. Afterwards, the harvested cells were washed with serum-free culture medium. ROS released from mRTECs were measured by a fluorescence microscope (Nikon Eclipse Ti, Japan) and followed by the determination of fluorescence intensity. Levels of intracellular oxidative stress were reflected by mean fluorescence intensity of dichlorofluorescein (DCF) as described.

To measure the amount of lipid peroxidation, mRTECs were collected for detection of superoxide dismutase (SOD) and malondialdehyde (MDA) activities using specific assay kits (Beyotime) according to the manufacturer's instructions.⁵

2.9 | Flow cytometry

Flow cytometry was used to assess the membrane and nuclear events during apoptosis. The H/R mRTECs, following their co-culture with

GDNF-AFSCs and AFSCs, were harvested by trypsinization after 3 days and washed twice in cold PBS. Next, cells were suspended in 500 μ L binding buffer containing 5 μ L Annexin V-FITC and 5 μ L PI (propidium iodide) (KeyGEN, Nanjing, China), gently mixed with a pipette and incubated at room temperature for 15 minutes. The apoptotic rate was obtained from the percentage of cells which were double-stained by Annexin V and PI detected by a flow cytometry (Becton-Dickinson, USA).

2.10 | Evaluation of caspase-3 activity

Relative caspase-3 activity was determined with a caspase-3 assay kit (Beyotime). Briefly, following treatment with paraffin for 90 minutes in 96-well plates, mRTECs were co-cultivated with GDNF-AFSCs or AFSCs in the transwell plates as described before. mRTECs were harvested by incubating 10 μ L protein of cell lysate per sample in 80 μ L reaction buffer containing 10 μ L caspase-3 substrate (Ac-DEVD-pNA) (2 mmol/L). Lysates were incubated at 37°C for 2 hours. Samples were measured spectrophotometrically at a wave length of 405 nm using Colorimetric Assay kits from R&D Systems (Thermo Scientific, Rockford, IL, USA).

2.11 | Western blot analysis

Cells were harvested and the whole proteins were extracted using protein lysis solution containing RIPA buffer and 1% phenyl methane sulfonyl fluoride (PMSF) (Beyotime, Jiangsu, China) according to the manufacturer's protocols. Identical amounts of protein samples were separated by SDS-PAGE, blotted onto a PVDF membrane (Millipore MA, USA). After blocking with 5% non-fat milk for 2 hours at room temperature, the membranes were incubated with antibodies against Gp91-phox, nitrotyrosine, Bax, Bcl-2 Abcam, Cambridge, MA, USA and β -actin (Santa Cruz Biotech, Santa Cruz, CA) at 4°C overnight. Then, the blots were washed with Tris Buffered Saline with Tween-20 for 3 times. Afterwards, goat anti-rabbit IgG or goat anti-mouse IgG (Santa Cruz Biotech, Santa Cruz, CA) were added for 2 hours at room temperature. The bands of interest were visualized by BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Nanjing, China). Western blot results were normalized to the expression of β -actin and analysed using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.12 | Statistical analysis

All data are expressed as mean values with standard deviations (mean $\pm$ SD). Differences between groups were assessed using Student's *t* test and 1-way analysis of variance (ANOVA) in SPSS 22.0 software (SPSS, Chicago, IL, USA). *P* < .05 was considered statistically significant.

3 | RESULTS

3.1 | AFSC extraction

After the initial plating of cells from the human amniotic fluid, few fibroblast-like cells attached to the surface of the 25-cm² tissue

FIGURE 1 Culture of amniotic fluid-derived stem cells (AFSCs) and lentivirus vector transfection. Morphology of AFSCs under high magnification of 3 passages. (A) AFSCs presented fibroblastic or needle shape with larger nuclei (200 $\times$). (B-D) Fluorescence expression of lentivirus vector with GDNF transfected AFSCs at 24, 48 and 72 hours. A small expression of 24 hours, along with the transfection continued, green fluorescent protein (GFP) expression gradually increased. For 72 hours after transfection, approximately 80% of AFSCs expressed GFP gene which shows green fluorescence (200 $\times$)

culture flasks within 3 days, with no visible directionality when cultured to 90% confluence (Figure 1A).

3.2 | Lentivirus transfection

Lentivirus vectors were constructed by Shanghai Jikai Gene Technology Con, and a MOI of 20 was used. Fluorescence microscopy was performed to observe GFP, which was indicative of transfection efficiency. Faint green fluorescence was observed after transfection on the first day. Fluorescence was obviously enhanced by the fifth day (Figure 1B-D).

3.3 | Modulation of growth factor expression following co-culturing

Having determined that GDNF-engineered AFSCs upregulated some growth factors,²⁷ we further investigated whether expression level of growth factors in the paraffin-treated mRTECs could increase with the help of GDNF-AFSCs. Our qRT-PCR data showed that the basal expression level of the bFGF and hepatocytes growth factor (HGF) were quite high (Figure 2). The basal expression level of the bFGF and HGF were quite high, whereas after the treatment of paraffin for 90 minutes, expression of HGF and bFGF in mRTECs were downregulated obviously ($P < .05$). Nevertheless, following their co-culturing with AFSCs or GDNF-AFSCs for 3 days, the expression of FGF and bHGF genes increased remarkably, especially in the GDNF-AFSCs group ($P < .05$), suggesting that the cytoprotective effects of GDNF-AFSCs may be attributed to the induction of these growth factors in injured mRTECs.

3.4 | Co-culture of GDNF-AFSCs modified mRTECs oxidative stress

In order to verify the anti-oxidation effect of GDNF-AFSCs on damaged mRTECs, the activities of SOD in mRTECs were detected. Following

FIGURE 2 Expression of growth factors released from the mRTECs following treatment with H/R. mRTECs were treated with paraffin before being co-cultured with amniotic fluid-derived stem cells (AFSCs) and GDNF-AFSCs. A, Quantitative assessment of growth factors by real-time PCR: Results showed upregulation of the growth factors in paraffin-injured mRTECs that co-cultured with GDNF-AFSCs compared to the AFSCs Cont: without paraffin treatment. (* $P < .05$ vs Control group; # $P < .05$ vs H/R group; $\Delta P < .05$ vs AFSCs group)

the treatment of paraffin, the level of SOD was dramatically decreased ($P < .05$). However, AFSCs and GDNF-AFSCs reversed the changes to the extent and there was no significant difference between the 2 groups ($P > .05$, Figure 3F), which supported the notion that AFSCs have the ability to mitigate the toxicity of oxidative stress. For the purpose of further assessing antioxidant potent of GDNF, MDA was applied for experiment (Figure 3G). The concentration of MDA was quite high at the site of lesion compared with the control group ($P < .05$). After co-culturing for 3 days, GDNF-AFSCs did play a more prominent role in repressing endogenously the release of MDA than AFSCs ($P < .05$). Interestingly, similar trend appeared on the ROS production according to the results of DCF fluorescence (Figure 3A-D). Moreover, we observed the changes of Gp91-phox and nitrotyrosine at the protein level assessed by Western blot (Figure 3H,I). It is apparent that the expression of Gp91-phox and nitrotyrosine on normal renal tubular epithelial cells is very weak. After

FIGURE 3 Glial-derived neurotrophic factor-amniotic fluid-derived stem cells (GDNF-AFSCs) prevented mRTECs oxidative stress and lipid peroxidation after H/R. mRTECs were treated with paraffin before being co-cultured with AFSCs and GDNF-AFSCs. (A) The intracellular reactive oxygen species (ROS) level as indicated by DCF fluorescence and measured by fluorescence microscopy upon DCF staining (400×). (B) ROS production was elevated in H/R group and decreased in AFSCs and GDNF-AFSCs groups, especially in GDNF-AFSCs group. (C) Superoxide dismutase (SOD) activity determined by SOD activity assay kit. The level of SOD was dramatically decreased in H/R group, However, AFSCs and GDNF-AFSCs reversed the changes and there was no significant difference between the 2 groups. (D) Malondialdehyde (MDA) concentration measured by the Thiobarbituric Acid Reactive substances (TBARS) assay. The concentration of MDA was increased in H/R group compared with control group, while reduced in AFSCs compared with H/R group, and further in GDNF-AFSCs group. (E,F) Western blot analysis of nitrotyrosine (NT), Gp91-phox. The expressions of NT, Gp91-phox were elevated in H/R group, whereas decreased in AFSCs group, and further decreased in GDNF-AFSCs group. Cont: without paraffin treatment. (* $P < .05$ vs Control group; # $P < .05$ vs H/R group; $\Delta P < .05$ vs AFSCs group)

being exposed to H/R, Gp91-phox and nitrotyrosine at day 1 were significantly increased, and there was no significant difference between H/R group and AFSCs group ($P > .05$), whereas their expression was significantly inhibited in GDNF-AFSCs group ($P < .05$). Meanwhile, the expression of Gp91-phox and nitrotyrosine at day 3 in H/R group was significantly increased ($P < .05$), while was decreased in AFSCs and GDNF-AFSCs groups ($P < .05$), especially in GDNF-AFSCs group ($P < .05$).

3.5 | Co-culture of GDNF-AFSCs restrained H/R-induced mRTECs apoptosis

What we have hypothesized is that the benefits of GDNF-AFSCs are related to the reduction of cellular oxidative stress, enhancement of the mitochondrial membrane potential and subsequent reversal of apoptosis.

To confirm the above speculation, the expression of anti-apoptotic Bcl-2 at day 1 in H/R group and AFSCs group were markedly decreased compared to that of the normal mRTECs as shown in Figure 4G and there was no significant difference between these 2 groups ($P > .05$), but their expression was significantly increased in GDNF-AFSCs group compared to that of the H/R group ($P < .05$). Meanwhile, the expression of Bcl-2 at day 3 in H/R group was significantly decreased ($P < .05$), while they were increased in AFSCs and GDNF-AFSCs groups ($P < .05$), especially in GDNF-AFSCs group ($P < .05$). On the contrary, mRTECs subjected to H/R injury responded with a notable increasing expression of Bax at day 1 and 3 in H/R group ($P < .05$), an indication of significant cell damage. While they were significantly decreased in AFSCs and GDNF-AFSCs groups ($P < .05$, Figure 4G,H), what is more, GDNF-AFSCs-treated group had a better effect on inhibiting the expressions of key apoptotic

FIGURE 4 Glial-derived neurotrophic factor-amniotic fluid-derived stem cells (GDNF-AFSCs) reduced mRTECs apoptosis after H/R. mRTECs were treated with paraffin before being co-cultured with AFSCs and GDNF-AFSCs followed by detection of apoptosis. (A) Cell apoptosis was examined by Annexin V-FITC/PI staining using flow cytometry. (B) AFSCs and GDNF-AFSCs significantly reduced mRTECs apoptosis induced by H/R, especially GDNF-AFSCs. (C) Detection of apoptotic activity using caspase-3 assay kit. The highest level of the activated caspase-3 was seen in paraffin-treated mRTECs, while AFSCs and GDNF-AFSCs decreased the levels of the activated caspase-3, especially GDNF-AFSCs. (D,E) Western blot analysis of Bax and Bcl-2. The expressions of Bax were elevated in H/R group and decreased in AFSCs and GDNF-AFSCs groups, especially in GDNF-AFSCs group. The expression of Bcl-2 in H/R group was markedly decreased, while they were increased in AFSCs and GDNF-AFSCs groups, especially in GDNF-AFSCs group. Cont: without paraffin treatment. (* $P < .05$ vs Control group; # $P < .05$ vs H/R group; $\Delta P < .05$ vs AFSCs group)

proteins ($P < .05$) which is in accordance with the captivity of caspase-3, an important step towards evaluation of cell apoptosis, analysed by a caspase-3 assay kit (Figure 4F). Besides, assessment of apoptotic cells by flow cytometry was further tested (Figure 4E). Most apoptotic mRTECs appeared in the H/R group (Figure 4B), while the control group is least ($P < .05$, Figure 4A). Moreover, compared with AFSCs group, apoptotic mRTECs in GDNF-AFSCs group significantly reduced ($P < .05$, Figure 4C,D). All these data suggest that GDNF-AFSCs have a protective effect against H/R-induced apoptosis in mRTECs.

4 | DISCUSSION

Renal ischaemia reperfusion injury is an unavoidable process and is acknowledged as being involved in acute or chronic kidney transplant

dysfunction. The principal goal of this study was to demonstrate the effect of GDNF-AFSCs on the RIRI and evaluating its possible mechanisms. An in vitro H/R model was generally employed to mimic renal injury microenvironment. And a co-culture system was established to assess repair potential of GDNF-AFSCs. In the current study, GDNF-AFSCs treatment dramatically counteracted oxidative/nitrative stress, alleviated apoptosis, and increased the growth factors within damaged cells, suggesting that GDNF-AFSCs represent an advantage for cell therapy for kidney injury.

Superoxide dismutase, regarded as a vital antioxidant enzyme in organisms, is the first material of biological removal of free radicals in the body, which was associated with a reciprocal increase in MDA level and ROS. MDA is one of the most important products of membrane lipid peroxidation which aggravates the damage of membrane. Recent studies revealed that a multicomponent phagocyte-type NADPH oxidase

is a major source of ROS production in many non-phagocytic cells including renal mesangial cells.²⁹ Gp91-phox is a core regulatory subunit of NADPH oxidase.³⁰ It is reported that when the balance between the anti-oxidative and oxidative status was broken by oxygen, large numbers of ROS may activate mitochondrial stress pathways to cause mitochondrial injury.³¹ Moreover, the production of NO probably leads to the cellular damage. Nitrotyrosine³² is now recognized as the marker of Peroxynitrite anion (ONOO) in the body. A considerable amount of literature shows that ONOO, as a product of NO and O₂ quick reaction, has obvious anti-oxidative capacity, which can inactivate enzyme activity, cause cell metabolism disorder, interfere with the mitochondria electron transfer and result in the membrane lipid peroxidation.³³

This paper provided results that the level of SOD fell down and MDA increased in the mRTECs after H/R. Nevertheless, by establishing the co-culture system, we found that GDNF-AFSCs exerted pronounced cytoprotective effect on suppressing MDA and ROS production and increasing the activity of SOD. Additionally, it was found that the expression of Gp91-phox and nitrotyrosine in GDNF-AFSCs-treated group decreased both at day 1 and 3, which made us suppose that GDNF-AFSCs attribute to suppress activation of NADPH oxidase directly or indirectly and partially alleviating the NADPH oxidase-dependent production of ROS. Khurana and Gajbhiye pointed out that AFSCs can ameliorate rotenone-induced oxidative stress model of Parkinson's disease by positively regulating the antioxidant-prooxidant mechanism.³⁴ Similar to our observation, GDNF-AFSCs may increase ROS elimination to normalize the imbalance between the anti-oxidative and oxidative status after H/R, which is beneficial to maintaining mitochondrial integrity.

Mitochondria are considered vital for integrity of cells, and any disruption in morphology and function of mitochondria can activate the intrinsic apoptotic pathway. Bax and Bcl-2 are known as the pro-apoptotic protein and anti-apoptotic protein, whose relative expression determines the cell survival and death.^{35,36} Caspase family is the main executor and mediator in the process of apoptosis, and its activation and overexpression can cause cell apoptosis. Caspase-3³⁷ is considered to be the most crucial and the terminal effector enzyme in apoptosis pathway. Under some special circumstances, the mitochondria are impaired and their membrane permeability transport holes open. After releasing from mitochondria, cytochrome C is combined with apoptotic protease activating factor 1, resulting in the production of apoptotic body. In this case, caspase-9 is activated, inducing the activity of the effector protein caspase-3, which can make the apoptosis into irreversible stage.³⁸ As expected, the apoptosis mechanism of mRTECs has been started under the action of paraffin. The balance between Bax and Bcl-2 was disrupted, and in turn triggered the caspase cascade. While we observed that GDNF-AFSCs was likely to prevent the downward spiral of mitochondrial dysfunction and cell apoptosis by restoring the balance of Bax and Bcl-2 as well as depressing caspase-3 viability.

Multiple studies have identified that AFSCs possess the potential of antioxidant, free radical-scavenging activities, anti-apoptosis ability and reducing calcium overload caused by hypoxia. Intriguingly, the therapeutic effect of AFSCs could be enhanced by pre-treatment GDNF, which increased stem cells homing to the tubule interstitial compartment. Our

experimental data also showed that GDNF-AFSCs treatment significantly reduced ONOO production, ROS generation and cell apoptosis as evidenced by the decreased levels of MDA, nitrotyrosine, Gp91-phox, Bax and caspase-3, as well as the increased levels of SOD and Bcl-2. According to these findings, we propose that GDNF-AFSCs are likely to have something to do with the endogenous antioxidant system via inhibiting the production and accumulation of ONOO and ROS, thus reducing mitochondrial stress-dependent apoptosis.

In a handful of *in vitro* studies, the H/R model could be a useful tool to simulate the situation of AKI *in vitro*. The co-culture system described here was used to separate cells from each other and only allow the secretion of cytokines to pass by, which forcibly proved that the interaction between the cells was derived from the paracrine mechanism. In a study by Prasongchean et al, AFSCs, as a rich source of factors acting in a paracrine manner, prevented cell death mainly through their secreted growth factors, rather than their differentiation and integration into the tissue.³⁹ As we have supposed, the repair effect of GDNF-AFSCs on the damaged mRTECs might put down to delivery of secreted trophic factors at high concentrations,⁴⁰ even if the underlying mechanism is still not completely clear.

The survival and growth of AFSCs need relatively favourable microenvironment. When the tissue is damaged, the environment of ischaemia or hypoxia is not conducive to cell survival and secretion of growth factors. Therefore, what we need to explore next is whether GDNF can inhibit the apoptosis of AFSCs and promote their proliferation and differentiation in animal experiments. Additionally, the effect of GDNF-AFSCs on the RIRI *in vivo* remains unknown, which is imperative to be further verified.

Our study showed that in a co-culture experimental model, the level of paraffin-induced ROS was significantly reduced in mRTECs, especially with the help of GDNF-AFSCs, which could get rid of oxygen free radicals and restrain cell apoptosis. Further studies are required to fully understand the underlying mechanisms, which will help us to put GDNF-AFSCs into practice and develop strategies for treating kidney disease.

AUTHORS' CONTRIBUTIONS

J.W: collection and/or assembly of data, data analysis and interpretation, and manuscript writing; F-ZW and Z.-J.W: collection and/or assembly of data, data analysis and interpretation; S.-L.L and L.C: collection and/or assembly of data; C.-X.L.: administrative support and financial support; D.S.: conception and design, and final approval of manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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REFERENCES

- Xue JL, Daniels F, Star RA, et al. Incidence and mortality of acute renal failure in Medicare beneficiaries, 1992 to 2001. *J Am Soc Nephrol*. 2006;17:1135-1142.
- Zhao WY, Han S, Zhang L, Zhu YH, Wang LM, Zeng L. Mitochondria-targeted antioxidant peptide SS31 prevents hypoxia/reoxygenation-induced apoptosis by down-regulating p66Shc in renal tubular epithelial cells. *Cell Physiol Biochem*. 2013;32:591-600.
- Giraud S, Favreau F, Chatauret N, Thuillier R, Maiga S, Hauet T. Contribution of large pig for renal ischemia-reperfusion and transplantation studies: the preclinical model. *J Biomed Biotechnol*. 2011;2011:532127.
- Kloner RA, Przyklenk K, Whittaker P. Deleterious effects of oxygen radicals in ischemia/reperfusion. Resolved and unresolved issues. *Circulation*. 1989;80:1115-1127.
- Zhong D, Wang H, Liu M, et al. Ganoderma lucidum polysaccharide peptide prevents renal ischemia reperfusion injury via counteracting oxidative stress. *Sci Rep*. 2015;5:16910.
- Cananzi M, De Coppi P. CD117(+) amniotic fluid stem cells state of the art and future perspectives. *Organogenesis*. 2012;8:77-88.
- De Coppi P, Bartsch G Jr, Siddiqui MM, et al. Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol*. 2007;25:100-106.
- Gekas J, Walther G, Skuk D, Bujold E, Harvey I, Bertrand OF. In vitro and in vivo study of human amniotic fluid-derived stem cell differentiation into myogenic lineage. *Clin Exp Med*. 2010;10:1-6.
- de Lara Janz F, Favero GM, Bohatch MS Jr, Aguiar Debes A, Bydlowski SP. Simvastatin induces osteogenic differentiation in human amniotic fluid mesenchymal stem cells (AFMSC). *Fundam Clin Pharmacol*. 2014;28:211-216.
- Gao Y, Connell JP, Wadhwa L, Ruano R, Jacot JG. Amniotic fluid-derived stem cells demonstrated cardiogenic potential in indirect co-culture with human cardiac cells. *Ann Biomed Eng*. 2014;42:2490-2500.
- Preitschopf A, Schorhofer D, Kinslechner K, et al. Rapamycin-induced hypoxia inducible factor 2A is essential for chondrogenic differentiation of amniotic fluid stem cells. *Stem Cells Transl Med*. 2016;5:580-590.
- Moorefield EC, McKee EE, Solchaga L, et al. Cloned, CD117 selected human amniotic fluid stem cells are capable of modulating the immune response. *PLoS ONE*. 2011;6:e26535.
- Segers VF, Lee RT. Stem-cell therapy for cardiac disease. *Nature*. 2008;451:937-942.
- Hauser PV, De Fazio R, Bruno S, et al. Stem cells derived from human amniotic fluid contribute to acute kidney injury recovery. *Am J Pathol*. 2010;177:2011-2021.
- Sedrakyan S, Da Sacco S, Milanesi A, et al. Injection of amniotic fluid stem cells delays progression of renal fibrosis. *J Am Soc Nephrol*. 2012;23:661-673.
- Rosner M, Schipany K, Gundacker C, et al. Renal differentiation of amniotic fluid stem cells: perspectives for clinical application and for studies on specific human genetic diseases. *Eur J Clin Invest*. 2012;42:677-684.
- Yao WQ, Hu QY, Ma YH, et al. Human adipose-derived mesenchymal stem cells repair cisplatin-induced acute kidney injury through antiapoptotic pathways. *Exp Ther Med*. 2015;10:468-476.
- Sun D, Bu L, Liu C, et al. Therapeutic effects of human amniotic fluid-derived stem cells on renal interstitial fibrosis in a murine model of unilateral ureteral obstruction. *PLoS ONE*. 2013;8:e65042.
- Baulier E, Favreau F, Le Corf A, et al. Amniotic fluid-derived mesenchymal stem cells prevent fibrosis and preserve renal function in a preclinical porcine model of kidney transplantation. *Stem Cell Transl Med*. 2014;3:809-820.
- Perin L, Sedrakyan S, Giuliani S, et al. Protective effect of human amniotic fluid stem cells in an immunodeficient mouse model of acute tubular necrosis. *PLoS ONE*. 2010;5:e9357.
- Rosner M, Hengstschlager M. Amniotic fluid stem cells and fetal cell microchimerism. *Trends Mol Med*. 2013;19:271-272.
- Duarte EP, Curcio M, Canzoniero LM, Duarte CB. Neuroprotection by GDNF in the ischemic brain. *Growth Factors*. 2012;30:242-257.
- Fon D, Al-Abboodi A, Chan PPY, et al. Effects of GDNF-loaded injectable gelatin-based hydrogels on endogenous neural progenitor cell migration. *Adv Healthc Mater*. 2014;3:761-774.
- Morigi M, De Coppi P. Cell therapy for kidney injury: different options and mechanisms—mesenchymal and amniotic fluid stem cells. *Nephron Exp Nephrol*. 2014;126:59.
- Zhang R, Lu Y, Li J, et al. Glial cell line-derived neurotrophic factor induced the differentiation of amniotic fluid-derived stem cells into vascular endothelial-like cells in vitro. *J Mol Histol*. 2016;47:9-19.
- Jezierski A, Rennie K, Zurakowski B, et al. Neuroprotective effects of GDNF-expressing human amniotic fluid cells. *Stem Cell Rev*. 2014;10:251-268.
- Rota C, Imberti B, Pozzobon M, et al. Human amniotic fluid stem cell preconditioning improves their regenerative potential. *Stem Cells Dev*. 2012;21:1911-1923.
- Halabian R, Roudkenar MH, Jahanian-Najafabadi A, Hosseini KM, Tehrani HA. Co-culture of bone marrow-derived mesenchymal stem cells overexpressing lipocalin 2 with HK-2 and HEK293 cells protects the kidney cells against cisplatin-induced injury. *Cell Biol Int*. 2015;39:152-163.
- Li JM. ROS generation by nonphagocytic NADPH oxidase: potential relevance in diabetic nephropathy. *J Am Soc Nephrol*. 2003;14:S221-S226.
- Kang IS, Kim C. NADPH oxidase gp91phox contributes to RANKL-induced osteoclast differentiation by upregulating NFATc1. *Sci Rep*. 2016;6:38014.
- Huttemann M, Helling S, Sanderson TH, et al. Regulation of mitochondrial respiration and apoptosis through cell signaling: cytochrome c oxidase and cytochrome c in ischemia/reperfusion injury and inflammation. *Biochem Biophys Acta*. 2012;1817:598-609.
- Rochette L, Lorin J, Zeller M, et al. Nitric oxide synthase inhibition and oxidative stress in cardiovascular diseases: possible therapeutic targets? *Pharmacol Ther*. 2013;140:239-257.
- Gao F. Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. *Circulation*. 2002;105:1497-1502.
- Khurana N, Gajbhiye A. Ameliorative effect of Sida cordifolia in rotenone induced oxidative stress model of Parkinson's disease. *Neurotoxicol*. 2013;39:57-64.
- Borkan SC. The role of BCL-2 family members in acute kidney injury. *Semin Nephrol*. 2016;36:237-250.
- Soriano Maria E, Scorrano L. Traveling back and forth from mitochondria to control apoptosis. *Cell*. 2011;145:15-17.
- Kavanagh E, Rodhe J, Burguillos MA, Venero JL, Joseph B. Regulation of caspase-3 processing by cIAP2 controls the switch between pro-inflammatory activation and cell death in microglia. *Cell Death Dis*. 2014;5:e1565.
- Ola MS, Nawaz M, Ahsan H. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. *Mol Cell Biochem*. 2011;351:41-58.
- Prasongchean W, Bagni M, Calzarossa C, De Coppi P, Ferretti P. Amniotic fluid stem cells increase embryo survival following injury. *Stem Cells Dev*. 2012;21:675-688.
- Skardal A, Mack D, Kapetanovic E, et al. Bioprinted amniotic fluid-derived stem cells accelerate healing of large skin wounds. *Stem Cells Transl Med*. 2012;1:792-802.

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