Neuroprotective effects of adipose-derived stem cells on ferrous sulfate-induced neurotoxicity

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KEYWORDS
Cytokines, mesenchymal stromal cells, paracrine communication, stroke

ABSTRACT
Background: Ferrous ion, a degradation product of hematomas, induces inflammatory reactions and other secondary injuries after intracerebral hemorrhage (ICH). Our study aimed to investigate the specific neuroprotective mechanism of adipose-derived stem cells (ADSCs) on ferrous ion-induced neural injury in vitro.

Methods: ADSCs were co-cultured with primary cortical neurons in a transwell system treated with ferrous sulfate to generate an in vitro ICH model. ADSCs and cortical neurons were cultured in the upper and lower chambers, respectively. Neuron apoptosis was determined by flow cytometry. The levels of insulin-like growth factor-1 (IGF-1), malondialdehyde (MDA) and nitric oxide synthase (NOS) activity in neuron culture medium were detected with commercial kits. In neurons, protein expression in phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) signaling pathway, nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway and apoptosis-related proteins were detected by western blot.

Results: ADSCs attenuated neural apoptosis, reduced MDA levels and NO activity induced by ferrous sulfate. In neurons, IGF-1 was increased, as were p-PI3K, p-Akt, Nrf2, HO-1, and Bcl-2 while cleaved caspase 3 was down-regulated.

Conclusions: ADSCs exert neuroprotective effects against ferrous ion-induced neuronal damage by secretory IGF-1 and increasing the levels of Akt-dependent Nrf2/ARE signaling pathway.

1 Introduction

Intracerebral hemorrhage (ICH) is one of the most devastating diseases having high morbidity and mortality, thus becoming the focus of global concern [1]. A series of inflammatory reactions, oxidative stress, and apoptosis triggered by hematoma degradation products
(e.g., ferrous iron) are responsible for poor outcomes [2]. In our study, we aimed to treat primary cortical neurons with ferrous sulfate (FeSO₄) to mimic iron release after ICH in vivo.

After ICH, neuron death is unavoidable and irreversible. Neuron Protection is indeed very significant for functional recovery [3]. Adipose-derived stem cells (ADSCs) are a type of fibroblast-like, multipotent mesenchymal stem cells, which can be easily isolated from the adipose tissue and highly proliferate in vitro. ADSCs have been shown to improve neurological deficits through directional neural differentiation and reduction of inflammatory reactions in rodent stroke models [4]. In addition, they secrete several cytokines, e.g. insulin-like growth factor-1 (IGF-1), which play a biological role in wound healing, cell migration, and differentiation [5]. Our previous study demonstrated that ADSCs transplantation decreased cell apoptosis in vivo [6]. However, whether ADSCs have protective effects on FeSO₄-injured neurons remains unclear. IGF-1 is a natural and effective activator of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway and participates in cell proliferation, growth, survival, and angiogenesis [7]. Nitric oxide (NO) is a cytotoxic effector which may induce tissue injury, and nitric oxide synthase (NOS) represents a crucial enzyme controlling NO synthesis [8]. NOS activity can reflect the inflammatory levels in the organism [9]. Nuclear factor erythroid 2-related factor 2 (Nrf2) not only plays a significant role in cellular anti-oxygen stress, but also regulates stem cell proliferation and differentiation by regulating the levels of reactive oxygen species (ROS) [10]. Its downstream molecule heme oxygenase-1 (HO-1) is a rate-limiting enzyme in the production of iron ions during hemoglobin degradation. After ICH, iron overproduction is associated with HO-1 induction in the perihematomatoma area [11]. The present study aimed to demonstrate the neural protective effects of ADSCs associated with ferrous-induced pathologic variations.

2 Material and methods

2.1 Cell isolation and preparation

All animal experiments were approved by the Experimental Animal Ethical Committee of Tongji Hospital (IRB ID: TJ-A20170702). Cortical neurons were prepared from fetal Sprague-Dawley (SD) rats (at an embryonic age of 16–17 days; Animal Center of Tongji Medical College, Huazhong University of Science and Technology). Fetal rats were removed from three pregnant rats by cesarean section while anesthetized with 2% pentobarbital sodium (Sigma-Aldrich, St. Louis, MO, USA). Briefly, fetuses were removed, decapitated, and stripped of the meninges. Cortical tissues were cut into pieces and digested in Hanks balanced salt solution with 0.125% trypsin (Invitrogen Corp., CA, USA) for 15 min at 37 °C. After dissociation with a fomed Pasteur pipette in Dulbecco’s modified eagle medium/nutrient mixture F-12 (DMEM-F12; Gibco Co., CA, USA) containing 10% fetal bovine serum (FBS; Gibco Co., CA, USA), cells were seeded on poly-L-lysine-coated culture plates and incubated at 37 °C in a humidified 5% CO₂ atmosphere. The medium was replaced after 4 h and thereafter, half of the medium was refreshed every 3 days with Neurobasal Medium (Gibco Co., CA, USA) supplemented with 2% B27, 1% penicillin-streptomycin, and 1% L-glutamine (Sigma-Aldrich Co., MO, USA). All pregnant rats were sacrificed by tail vein injection of 2 mL of air.

ADSCs were isolated from non-pregnant female SD rats (4–6 weeks old, 100–160 g;
Animal Center of Tongji Medical College. Female SD rats were sacrificed by neck dislocation. The adipose tissue in the inguinal area was rapidly removed under sterile conditions and cut into pieces. Fragments were incubated in phosphate buffer solution (PBS; HyClone, USA) containing 0.1% collagenase I (Invitrogen Corp., CA, USA) for 45 min at 37 °C with gentle agitation. Suspensions were neutralized with an equal volume of DMEM-F12 (HyClone, USA) containing 10% FBS and centrifuged at 1100 rpm for 10 min. The pellet was resuspended with culture medium (DMEM-F12/10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine) and seeded in 25 cm² culture dishes (Corning, NY, USA). Twenty-four hours later, non-adherent cells were removed by changing the medium. Due to purity concerns, cells under generation 3–5 were not employed. The isolation and identification were modified from previous work [12, 13].

2.2 Establishment of co-culture systems

Co-culture systems were established in 6-well transwell plates (0.4 μm, Corning, NY, USA). Primary cortical neuron cells were placed in the lower chamber (2 × 10^⁶ cells per well) for 11 days. The upper chambers with ADSCs (1 × 10^⁶ cells) were inserted for 12 days. After 24 hours, the media in upper and lower chambers achieved a balanced state. Cultured primary cortical neurons were randomly assigned to the following four groups: vehicle (normal neurons), ADSCs (neurons co-cultured with ADSCs), FeSO₄ (neurons treated with FeSO₄), and ADSCs + FeSO₄ (neurons co-cultured with ADSCs and treated with FeSO₄) groups. Triplicate experiments were repeated for ≥3 times.

2.3 FeSO₄ exposure and cell survival assay

To obtain the optimal FeSO₄ concentration, the 3-(4,5-4imethylthiazol-2-yl)-2,5-diphenyltetrazoli-um bromide (MTT; Beyotime, China) assay to test cell viability after treatment with FeSO₄ was performed as described previously. Neurons were exposed to FeSO₄ at concentrations ranging from 0 to 20 μM. After 24 hours, 30 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h in the dark. Dimethyl sulfoxide (DMSO; Sigma-Aldrich Co., MO, USA) was then added to dissolve the formazan crystals. Absorbance was measured at 490 nm on the DG3022 Microplate Reader (Pharmacia Biotech Inc., USA).

2.4 Malondialdehyde and NOS assays

Malondialdehyde (MDA) levels were measured with the MDA assay kit (Beyotime, China) following manufacturer’s instructions. In addition, the cortical neuron culture medium was aspirated, and cells harvested in ice-cold radio immunoprecipitation assay lysis buffer (RIPA; Beyotime, China) containing phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich Co., MO, USA). After sonication, lysed cells were centrifuged at 12,000 rpm for 10 min to remove debris, and MDA levels in supernatants quantified at 532 nm. NOS activity in neurons was detected according to manufacturer’s instruction (A014-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Afterwards, neurons were harvested in ice-cold PBS and lysed by repeated freeze-thawing in liquid nitrogen. Samples were centrifuged at 12,000 rpm for 10 min to remove debris and NOS activity in supernatants quantified at 530 nm. Following manufacturer’s instructions, we determined total NOS (tNOS) and induced NOS (iNOS) activities, respectively. MDA levels and NOS activity were then normalized to protein amounts.
2.5 Cell apoptosis assay

Cell apoptotic rate was analyzed by flow cytometry with Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich Co., MO, USA). Briefly, harvested cells were washed three times and resuspended to a final density of 10^6/mL. Cell suspensions were subsequently stained with Annexin V-FITC and propidium iodide (PI) for 15 min at room temperature in the dark. This staining distinguished neurons into three subsets of cells: viable cells (Annexin V-FITC negative, PI negative), early apoptotic cells (Annexin V-FITC positive, PI negative), and late apoptotic or necrotic cells (Annexin V-FITC positive, PI positive). Apoptotic neurons were detected using a flow cytometer (Becton Dickinson Co., NJ, USA) and analyzed with the FlowJo software (version 7.6).

2.6 Immunofluorescence

Glass slides with ADSCs from the ADSCs and ADSCs + FeSO₄ groups in the upper chambers, were fixed with 4% paraformaldehyde for 30 min, incubated with 0.25% Triton X-100 for 20 min, and blocked with 5% bovine serum albumin (Invitrogen Corp., CA, USA) for 1 h. Samples were incubated with anti-HO-1 (1:200; Abcam, Cambridge, UK) primary antibodies overnight at 4 °C. Cells were then washed and incubated with rabbit anti-mouse IgG (1:300) at 37°C for 1 h and 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Samples were observed under a fluorescence microscope (Olympus Life Science, MA, USA). Fluorescence intensity was measured with the ImageJ 1.50b software developed by the National Institutes of Health, USA.

2.7 IGF-1 quantification by enzyme-linked immunosorbent assay (ELISA)

IGF-1 levels in the different groups were measured with the Rat IGF-1 Quantikine ELISA kit (R&D Systems Inc., MN, USA) based on manufacturer’s instructions.

2.8 Western blotting

The culture medium was aspirated, and cultured primary cortical neurons harvested. DM1A (1:1000; Sigma-Aldrich Co., MO, USA) was used in western blot analysis as loading control. All membranes were incubated overnight with the following primary antibodies: anti-pPI3K (1:500; Cell Signaling Technology, Inc., MA, USA), anti-pPI3K (1:1000; Cell Signaling Technology, Inc., MA, USA), anti-Nrf2 (1:300; Santa Cruz, USA), anti-HO-1 (1:500; Abcam, Cambridge, UK), anti-B-cell lymphoma-2 (Bcl-2) (1:1000; Abcam, Cambridge, UK), anti-Bcl-2-associated x protein (Bax) (1:1000; Abcam, Cambridge, UK), anti-Akt (1:1000; Cell Signaling Technology, Inc., MA, USA), anti-p-Akt (ser473) (1:1000; Cell Signaling Technology, Inc., MA, USA), anti-caspase 3 (1:500; Abcam, Cambridge, UK), and anti-cleaved caspase 3 (1:500; Abcam, Cambridge, UK). This was followed by incubation with secondary antibodies (1:2000; Cell Signaling Technology, Inc., MA, USA). Bands were visualized on an Odyssey Infrared Imaging System (LI-COR Biosciences Co., NE, USA) and quantitated with the ImageJ 1.50b software (National Institutes of Health, USA).

2.9 Statistical analysis

Statistical analyses were performed with the SPSS software (version 19.0). Data are expressed as mean ± standard deviation (SD). Multiple comparisons were performed by one-way analysis of variance with the Tukey–Kramer multiple comparisons post hoc test for parametric data as indicated, and we reanalyzed data without the need for t-tests. P < 0.05 was considered to indicate a statistically significant difference.
3 Results

3.1 ADSCs ameliorate ferrous sulfate-induced neuronal apoptosis in cultured primary neurons

The MTT assay showed that FeSO₄ induced dose-dependent inhibitory effects on neuron viability. In addition, we found 8 μM FeSO₄ to be the most effective concentration [Fig. 1(A)]. Then, 8 μM FeSO₄-treated neurons for time-course experiment showed that 24 hours was the optimal effect time [Fig. 1(B)] and therefore used in subsequent experiments.

Flow cytometry was used to quantify FeSO₄-induced neuronal apoptosis. As shown in Fig. 1(C), few apoptotic cells were detected in the vehicle and ADSCs groups. The FeSO₄ challenge increased neuronal apoptotic rate. When co-cultured with ADSCs, the apoptotic rate decreased significantly ($P < 0.05$ or $P < 0.01$).

3.2 ADSCs reduce ferrous sulfate-induced MDA production in cultured primary neurons

MDA is frequently used as an index of membrane lipid peroxidation and reflects cellular ROS levels. MDA levels in neurons were increased approximately 3-fold after the FeSO₄ challenge. As illustrated in Fig. 2(A), MDA levels were significantly decreased in the ADSCs + FeSO₄ group ($P < 0.01$), indicating that ADSCs exerted neuroprotective effects against FeSO₄-induced oxidative stress.

![Fig. 1](https://ms03.manuscriptcentral.com/brainsa) Cell viability and the effects of ADSCs on FeSO₄-treated neurons. (A) Dose-dependent FeSO₄ effects on neural cell viability. The dose for lethal concentration 50 (LC50) was 8 μM ($^*P < 0.05$ as compared with 0 μM group). (B) Cell viability of FeSO₄-treated neurons at different times. The longer the FeSO₄ treatment time, the lower the neuron viability, with 24 hours as the optimal effect time ($^*P < 0.05$ as compared with 2 h group). (C) Representative plot of flow cytometry on cell apoptosis and summarized data based on cell apoptotic rate in different groups. Few apoptotic cells were detected in the vehicle and ADSC groups. The FeSO₄ challenge increased neuronal apoptotic rates. When co-cultured with ADSCs, the apoptotic rate significantly decreased ($^*P < 0.05$, $^{**}P < 0.01$).

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3.3 Ferrous sulfate promotes IGF-1 secretion from ADSCs

We detected IGF-1 levels in culture medium (CM) of ADSCs and neurons, respectively. As shown in Fig. 2(B), IGF-1 levels were increased in FeSO₄-treated ADSCs-CM in comparison with the vehicle group. Similarly, in neuron-CM, IGF-1 levels were increased in the ADSCs co-incubation group compared with the vehicle group. IGF-1 levels in the ADSCs + FeSO₄ group were almost 2-fold higher than in FeSO₄-treated neurons [P < 0.05 or P < 0.01; Fig. 2(C)].

3.4 ADSCs suppress NOS activity in cultured primary neurons

NOS are widely distributed in endothelial cells, macrophages, neurophages and nerve cells and consists of three isozymes, namely neuronal NOS in normal conditions, endothelial NOS, and iNOS. Inappropriate iNOS overproduction and altered NO levels had been reported to contribute to the disruption of the balance between oxidation and anti-oxidation after ICH, ultimately triggering the inflammatory cascade. Therefore, we measured tNOS and iNOS activity in each group. The results showed extremely low tNOS and iNOS activities in primary neurons in vehicle and ADSCs groups. FeSO₄ significantly increased both tNOS and iNOS activities in the FeSO₄ group, which were reduced by co-culturing with ADSCs (P < 0.05). These findings demonstrated that ADSCs exerted antioxidative effects by
down-regulating antioxidant enzymes [Fig. 2(D)].

3.5 HO-1 expression correspondingly increased in ferrous sulfate-treated ADSCs

It has been shown that HO-1 has anti-inflammatory and antiapoptotic effects [14]. HO-1 expression could be accordingly increased in the stress state for self-protection. We assessed ADSCs’ HO-1 expression in the upper chambers. Immunofluorescent staining showed that HO-1 expression was markedly increased after treatment with FeSO₄ [P < 0.05; Figs. 3(A) and (B)].

3.6 PI3K/Akt and Nrf2/HO-1 were involved in antiapoptotic and antioxidant effects in primary neurons co-cultured with ADSCs

The PI3K/Akt pathway plays a critical role in cell survival and proliferation. For further mechanistic exploration, we analyzed the PI3K/Akt pathway in the primary neurons cultured in the lower chambers by western blot. Ferrous sulfate markedly reduced phospho-PI3K and phospho-Akt expressions in neurons which could explain the higher apoptotic rate in the FeSO₄ group. Phospho-PI3K and phospho-Akt significantly increased after co-culture with ADSCs (P < 0.05, vs. Vehicle group). Meanwhile, total PI3K, phospho-PI3K and phospho-Akt were significantly increased in the ADSCs + FeSO₄ group [P < 0.05 or P < 0.01, vs. FeSO₄ group; Fig. 4(A)].

Both Nrf2 and HO-1 were extremely low in primary neurons in the vehicle group not subject to stress. After FeSO₄ treatment, Nrf2 and HO-1 expression increased. ADSCs could promote the expression of proteins in the Nrf2/HO-1 pathway, an effect even stronger in the ADSCs + FeSO₄ group [P < 0.05 or P < 0.01; Fig. 4(B)].

To confirm the role of the PI3K/Akt and NRF2/HO-1 pathways, the apoptosis-related proteins CC3/cleaved-CC3 and Bax/Bcl-2 were examined as well. The Bcl-2 to Bax ratio and cleaved-CC3 expression were significantly increased in the ADSCs + FeSO₄ group compared with the FeSO₄ group [P < 0.05 or P < 0.01; Fig. 4(C)]. These results indicated that both pathways may contribute to the protective effect of ADSCs on FeSO₄-induced injuries in primary neurons.

4 Discussion

The current findings provide direct evidence that ADSCs exert neuroprotective effects by activating the PI3K/Akt pathway to induce Nrf2 nuclear translocation leading to increased HO-1 expression.
expression. Together providing protection against iron-induced oxidative damage in neurons.

Oxidative stress plays an important role in ICH [15]. Ferrous ions released from lysed erythrocytes after ICH are a strong reducing agent, which can easily lose electrons and be oxidized to ferric ion, thereby generating free oxygen radicals. Further, ferrous-induced neuronal death is critical for the pathogenesis of ICH [16, 17]. Our experimental results showed that iron-overload leads to irreversible damage to neuron cell membranes, followed by increased iron-catalyzed NOS activity, which eventually leads to neuron apoptosis.

ADSCs transplantation therapy is a reported promising therapeutic strategy for ICH [18]. Recently, ADSCs were reported to protect neurons against a variety of harmful stimuli [19]. In ICH research, our previous study demonstrated that ADSC transplantation decreased cell apoptosis; and that transplanted ADSCs could differentiate into neuron-like and astrocyte-like cells around the hematoma [6]. However, the specific mechanism remains unclear. ADSCs were reported to secrete bioactive levels of antiapoptotic growth factors such as BDNF, GDNF, VEGF [20], HGF, and IGF-1 [21]. In this study, we focused on IGF-1 and demonstrated that IGF-1 secreted by ADSCs played a key, if not the sole, role in protecting...
neurons from FeSO₄ toxicity. Next, we examined and confirmed that the downstream activated PI3K/Akt pathway participated in ADSCs-induced neuroprotection against FeSO₄ toxicity. This result was consistent with a previous report showing that IGF-1 was able to protect whole neurons [22].

Nrf2 is a major stress responder that activates antioxidant and cytoprotective genes through binding to antioxidant response element (ARE) motifs following its nuclear translocation [23]. The downstream protein HO-1 catalyzes heme breakdown into iron, carbon monoxide, and biliverdin, which is rapidly converted to bilirubin. Much controversy exists regarding the predominant effect of HO-1 in oxidative injury [24]. HO-1 may have pro-oxidative effects or the opposite, an antioxidant effect [25]. Thus, HO-1 effects in FeSO₄-mediated oxidative stress in neurons remain unclear. In our study, Nrf2 and HO-1 were hardly expressed in the vehicle group. When exposed to FeSO₄, Nrf2 and HO-1 levels were expected to drastically increase, as it was considered a beneficial stress response adapted to the harmful environment. According to previous studies, Nrf2 activation is triggered by protein kinase, oxidative stress generated ROS, or other electrophiles in the cytoplasm [26]. Based on the current study, Nrf2/HO-1 up-regulation might exert neuroprotective effects not only in association with FeSO₄ but also with the increased kinase levels (Akt and PI3K) in the ADSCs + FeSO₄ group. Our result was consistent with Regan’s result that heme-mediated injury to astrocytes was attenuated by the induction of endogenous HO-1 [27], but contrasted with Wang’s observations that an HO-1 knockout protects the striatum from ferrous iron-induced injury in a male-specific manner in mice [28]. These observations suggest that HO-1 effects on oxidative cell injury may considerably vary according to cell type and chemical properties. Cleaved caspase 3 as key apoptotic mediator was down-regulated in combination with increased antiapoptotic proteins Bcl-2 in the ADSCs + FeSO₄ group, which finally confirmed the protective effect of HO-1 in FeSO₄-induced neuron injury.

However, the present study still has a number of limitations. Specific agonists (Curcumin, Ezetimibe) and antagonists (ML385, Hinokitiol) would be useful for clarifying and validating the regulatory effects of ADSCs on the signaling pathways in this study. Further in vivo studies are required to verify the neuroprotective effect of ADSCs in ICH.

**Conflict of interests**

All contributing authors have no conflict of interests to declare.

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