Original Paper

Combination of Ginsenoside Rg1 and Bone Marrow Mesenchymal Stem Cell Transplantation in the Treatment of Cerebral Ischemia Reperfusion Injury in Rats

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Key Words

Ginsenoside Rg1 • BMSCs • Cerebral Ischemia Reperfusion • Protective roles

Abstract

Background/Aims: The present study aims to explore the protective role and mechanism of ginsenoside Rg1 combined with bone marrow mesenchymal stem cell (BMSC) transplantation for cerebral ischemia reperfusion injury (CIRI) in rat brain. **Methods:** One hundred twenty male SD rats were randomly divided into a sham group, an Ischemia Reperfusion (IR) group, an IR group treated with BMSC transplantation (IR+BMSCs), an IR group treated with Rg1 (IR+Rg1), and an IR group treated with BMSC transplantation and Rg1 (IR+Rg1+BMSCs). To establish a CIRI model, right middle cerebral artery embolization was used. The neurological score, 2,3,5-triphenyltetrazolium chloride monohydrate (TTC) staining and brain water content were detected to assess the treatment efficiency. HE staining and TUNEL were used to explore the pathologic changes and apoptosis. To explore the protein levels of neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP), immunofluorescence was utilized. Western blotting was used to explore apoptosis-related proteins such as Bcl-2 and Bax. **Results:** Compared with the sham group, the IR group demonstrated obvious ischemic changes, such as significant neurologic defects and enhanced brain water content. The Rg1 treatment resulted in an obvious decrease in cell apoptosis and improved ischemic conditions. By BMSC transplantation, the transplanted cells could be differentiated into neurons and glial cells, which also improved cerebral ischemia. More importantly, the IR+Rg1+BMSCs group

showed the best treatment efficiency with reduced cell apoptosis and better cerebral recovery.

Conclusions: The Rg1 treatment resulted in an obvious decrease in cell apoptosis, while the transplanted cells could be differentiated into neurons and glial cells, which also improved cerebral ischemia.

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Introduction

Cerebral ischemia is a major cause of morbidity and mortality in both developed and developing countries. Approximately 50% to 70% of survivors demonstrate a serious disability, such as paralysis and aphasia, which brings a heavy burden to the individual, family and society [1, 2]. Thus, it is important for researchers to study the specific mechanism of cerebral ischemia. Many pathological and physiological changes are involved in the process of cerebral ischemia injury, among which cell apoptosis is one of the most important [1]. Currently, the major treatment methods include thrombolysis, anticoagulation, application of a calcium channel regulator, and free radical scavenging. However, these methods could not realize the regeneration of neuron necrosis [3]. Thus, the problem of ischemic stroke disability and death cannot be fundamentally solved. It is important to explore new treatment methods in order to reduce cerebral injury, enhance neurologic recovery, and reduce disability and mortality.

To reduce the morbidity and mortality of ischemic encephalopathy, stem cell transplantation is increasingly applied to cure the disease. Bone marrow stromal cells (BMSCs) are characterized by good sources, excellent self-renewal capacity and quick *in vitro* amplification speed, which makes them a good option for cell transplantation [4, 5]. However, after transplantation, the survival rate of BMSCs is lower, a finding that may be related to the change in the microenvironment. In the circumstances of cerebral ischemia and anoxia, tissue-excited amino acids, oxygen-free radicals, adhesion molecules and inflammatory factor secretion are significantly enhanced, leading to alteration of the brain cell survival microenvironment, thereby affecting BMSC survival and proliferation [6, 7]. The low survival rate of BMSC transplantation greatly affects the treatment effect. Therefore, searching for a drug that can improve the survival rate of BMSCs has become a research hotspot.

Ginsenoside Rg1 is a traditional stem extract and is one of the main active ingredients of ginseng [8, 9]. It has been shown that as a small molecular substance, ginsenoside Rg1 easily passes through the blood brain barrier. Moreover, ginsenoside Rg1 can promote stem cell orientation transformation and induce stem cell proliferation [10, 11]. Currently, there are no reports on the combination of ginsenoside Rg1 and BMSCs in the treatment of ischemic encephalopathy. In this study, we combined Rg1 treatment and BMSC transplantation in order to study their roles in rat neuron recovery and explore the specific mechanisms involved in this process.

Materials and Methods

hBMSC Culture

HBMSCs/GFP was purchased from Cyagen Biosciences Inc. (RASM-01101, USA) and incubated in low-glucose Dulbecco's modified Eagle's medium (L-DMEM; Gibco, Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% CO₂. The medium was replaced with fresh medium every third day. When the hBMSCs reached 80% confluence, the cells were digested with 0.125% trypsinase and 0.01%. At passage 3, hBMSCs were collected and used for transplantation. The transfection efficiency was determined as GFP-expressing cells/all cells × 100%.

Establishing MCAO and reperfusion models

Transient MCAO was established using an intraluminal vascular occlusion method as previously described [12]. Briefly, rats were anesthetized with 3.5% chloral hydrate. A 18.5 ± 0.5 mm monofilament

nylon suture (0.265 mm diameter) was made, which advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the middle cerebral artery. For the sham group, the length of monofilament nylon suture was less than 15 mm. At 2 hours after MCAO, the nylon suture was withdrawn until the suture tip cleared the lumen of the external carotid artery to realize reperfusion.

Grouping

At 24 hours after MCAO, one hundred twenty rats were randomly divided into five groups: a sham + saline group ($n = 24$), an IR + saline group ($n = 24$), an IR + BMSC group ($n = 24$), an IR + Rg1 group ($n = 24$), and an IR + BMSCs group + Rg1 group ($n = 24$).

hBMSC transplantation and Rg1 treatment

At 24 hours after MCAO and neurological assessment, rats were anesthetized and received transplantation of hBMSCs or saline. The femoral vein was punctured under a microscope with a depth of 2.6 mm. Approximately 5.0×10^6 /ml hMSCs in 10 μ L of saline were injected into the animals at a rate of 1 μ L/min; an equal volume of saline was injected into animals at the same rate. The wounds were stitched up immediately after transplantation. Rg1 or saline was intraperitoneally injected (20 mg/kg) once per day at 24 h after operation.

Assessment of the neurological deficit score

The neurological deficit score was assessed at 24 h, 7d and 14d after reperfusion. Modified neurological severity scores were used [1]: 0, no motor deficits (normal); 18, the most severe deficits. The higher the score, the more severe the deficit was [13, 14]. If no deficit was observed following 24 h recovery post-anesthesia, the animal was excluded from further investigation.

Edema measurement

The rats were sacrificed by decapitation under deep anesthesia with 10% chloral hydrate at 14 d of reperfusion. The ipsilateral and contralateral hemispheres were dissected, and the wet weight of the tissues was determined. The tissues were then dried at 60°C for 6 h. The percentage of cerebral water was determined as follows: (wet weight - dry weight) / wet weight $\times$ 100%.

TTC staining

After 14 d of reperfusion, the rat brains were removed and frozen at -80°C for 5 min. Two-millimeter coronal slices were made using a rodent brain matrix. The sections were stained for 20 min at 37°C with 2% 2,3,5-triphenyltetrazolium chloride monohydrate (TTC; Sigma, Los Angeles, CA, USA) and then were fixed with 4% formalin. The sections were scanned, and the infarction area in each section was calculated by subtracting the non-infarct area of the ipsilateral side from the area of the contralateral side using CIAS-1000 analysis software. Infarction areas on each section were summed and multiplied by the section thickness to yield the total infarction volume [15].

Hematoxylin-eosin (H&E) staining

Brains were removed after 14 d of reperfusion and fixed in 4% paraformaldehyde at 4°C for 72 h, and then dehydrated and embedded in paraffin blocks. Coronal sections backward from the optic chiasma were cut at a thickness of 5 mm. The sections were deparaffinized and hydrated with decreasing concentrations of alcohol, stained with H&E, and photographed under a microscope (Carl ZeissA1; Carl Zeiss, Oberkochen, Germany).

TUNEL staining

TUNEL staining was performed according to the manufacturer's instructions (Roche, USA). DNase of 1500U/ml was used as the positive control, while TUNEL staining without TdT enzyme was used as the negative control. Apoptotic cells were photographed under a microscope (Carl ZeissA1). Three non-overlapping fields of view were randomly selected for each slice under 400 $\times$ magnification. The total cell number and number of apoptotic cells in each field were counted. The percentage of apoptotic cells was calculated as follows: (the number of apoptotic cells in each field / the total cell number in each field) $\times$ 100% [16].

Immunohistochemical staining

After washing them with PBS, sections were incubated with a blocking solution (10% normal goat serum, 0.3% Triton X-100 in PBS) for 30 min at 37°C. The slices were probed with primary anti-NSE polyclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GFAP polyclonal antibody (1:200; Santa Cruz Biotechnology) at 4°C by overnight incubation. After they were rinsed, sections were incubated with goat TRITC-conjugated anti-rabbit IgG (1:200; Sigma) for 1 h at 37°C. Slides were photographed using a microscope (Carl ZEISS A1) at 40× magnification using a standard procedure. PBS was used as the negative control for the primary antibody. Positive cells were counted using the CIAS-1000 cell image analysis system. The average positive cell number was counted from each perspective [17].

Western Blotting Analysis

Fifteen-milligram brain tissues were treated with 1 ml of RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl 1% (v/v) NP-40, 0.1% (w/v) SDS) containing 1% (v/v) PMSF (SolarBio, Beijing, China), 0.3% (v/v) protease inhibitor (Sigma) and 0.1% (v/v) phosphorylated proteinase inhibitor (Sigma). Next, the supernatants were extracted from the lysates after they were centrifuged at 12000 rpm at 4°C for 15 min. A BCA protein assay kit (Pierce, Rockford, IL, USA) was used to determine the relative concentration of the total proteins. The proteins were separated on an SDS-PAGE gel (10% (v/v) polyacrylamide) and transferred onto a PVDF membrane at 300 mA for 2 h. The membranes were incubated with anti-β-actin, Bcl-2 and Bax (CST, New Boston, TX, USA) overnight at 4°C. Next, the membranes were washed with TBST 4 times (5 min/time). HRP-conjugated goat anti-rabbit IgG (CST; 1:5000 dilution) was prepared, and the membranes were incubated with it for 2 h at room temperature. After incubation, the membranes were washed 4 times (5 min/time). Enhanced chemiluminescence (Millipore Corporation, Billerica, MA, USA) was then performed according to the manufacturer's recommendations. The target protein was normalized against β-actin [18].

Statistical analysis

All values were presented as the means ± SD. The data shown were analyzed for significance using Student's t-test or One Way ANOVA using SPSS13.0 software. A P value less than 0.05 was considered to be significantly different.

Results

Morphological observation of BMSCs/GFP

According to the microscopy, adherent cells grew faster and were tightly packed with uniform cell morphology and size (Fig. 1A). Fluorescence microscopy demonstrated obvious green fluorescence in BMSCs. The transfection efficiency of the cells reached 95% (Fig. 1C).

Assessment of neurological outcomes, brain edema and infarct volume

The sham group demonstrated no neurological deficits. In comparison, the other four groups showed neurological deficits 24 h post reperfusion (Fig. 2A). Compared with

Fig. 1. The morphological observation of BMSCs/GFP under the microscope (A) or fluorescence microscope (B). (C) Statistical analysis of the transfection efficiency. Quantitative data (n = 3) are given as the mean ± SD. *P<0.05 vs. control.

Fig. 2. Neurological outcomes (A), brain edema (B) and infarct volume (C, D) were assessed in the five different groups: sham group, IR group, IR + BMSCs group, IR + Rg1 group, and IR + BMSCs + Rg1 group. Statistical analysis of the transfection efficiency. Quantitative data ($n = 3$) are given as the mean $\pm$ SD. * $P < 0.05$ vs. sham group; $\Delta P < 0.05$ vs. IR group; * $P < 0.05$ vs. BMSC transplantation and Rg1 treatment group.

Fig. 3. Pathological changes in the left cerebral cortex (13 days after transplantation) were assessed using HE staining in the four different groups. $n = 6$ animals for each group.

the IR group, BMSC transplantation or Rg1 treatment significantly improved neurological deficits 7 d and 14 d post reperfusion. More importantly, the combination treatment group demonstrated the best effects (Fig. 2A). Compared with the sham group, the IR group demonstrated much higher brain water content. BMSC transplantation or Rg1 treatment significantly decreased brain edema. Additionally, combination treatment demonstrated the most obvious reduction of brain edema (Fig. 2B). Meanwhile, the infarct volume was significantly reversed in the BMSC transplantation or Rg1 treatment group compared with that in the IR group. In concert with the above results, combination treatment showed the best effects (Fig. 2C and 2D).

Fig. 4. Cell apoptosis was assessed in the rat cerebral cortex of the different groups (13 days after transplantation). n=6 animals in each group, (bar=10 μ m); * P <0.05 vs. sham group; Δ P <0.05 vs. IR group.

Fig. 5. Differential expression of NSE and GFAP was examined in different groups (13 days after transplantation). (A-B) The protein expression of NSE was assessed in the rat cerebral cortex of the different groups using immunofluorescence. Figure 5 (C-D) The protein expression of GFAP was assessed in the rat cerebral cortex of the different groups using immunofluorescence. n=6 animals in each group, (bar=10 μ m); * P <0.05 vs. sham group; Δ P <0.05 vs. IR group; # P <0.05 vs. BMSC transplantation and Rg1 treatment group.

Pathological changes of the left cerebral cortex in different groups

Normal brain structures were observed in the sham group. In comparison, the IR group demonstrated obvious changes in ischemia and hypoxia. Some cells were triangular or pyramidal, and the interstitial cells were loose (Fig. 3). More importantly, BMSC transplantation or Rg1 treatment alone or the combination treatment significantly reversed IR-induced ischemia and hypoxia changes.

Fig. 6. Protein levels of Bcl-2 and Bax were assessed in the five different groups (13 days after transplantation). (A) Western blot analysis was used to detect protein changes. Statistical analysis of the Bcl-2 (B) and Bax (C) levels. (D) The Bcl-2/Bax ratio was calculated in the different groups. n=6 animals in each group, * $P < 0.05$ vs. sham group; $\Delta P < 0.05$ vs. IR group; # $P < 0.05$ vs. BMSC transplantation and Rg1 treatment group.

Assessment of nerve cell apoptosis in the five different groups

Under fluorescence microscopy, BMSC transfected cells demonstrated green fluorescence, while the nuclei of apoptotic cells (TUNEL staining) showed red fluorescence. Hoechst staining was applied to indicate the cell nuclear. Next, the nuclei of apoptotic cells demonstrated a pink color after the images were merged. According to Fig. 4, few apoptotic cells were found in the sham group, while cell apoptosis reached 23%. In comparison, BMSC transplantation and/or Rg1 treatment significantly reduced cell apoptosis by nearly 10%.

Changes in NSE and GFAP expression in the different groups

Under fluorescence microscopy, BMSC transfected cells demonstrated green fluorescence, while TRITC-conjugated NSE and GFAP cells showed red fluorescence. According to Fig. 5A-5B, many NSE-expressing cells were observed in the sham group, while the IR group showed few NSE-positive cells. After BMSC transplantation or Rg1 treatment, NSE-positive cells obviously increased. More importantly, the combination treatment showed the best effects (Fig. 5A-5B).

Many GFAP-positive cells were also observed in the sham group. The IR group demonstrated few GFAP-positive cells. Compared with the IR group, GFAP-positive cells were increased in both the BMSC transplantation and Rg1 treatment groups. Additionally, the combination treatment group showed the best treatment effects (Fig. 5C-5D). These data indicated that the combination of BMSC transplantation and Rg1 treatment significantly enhanced the recovery of neuron cell injury.

Enhanced anti-apoptotic effects of BMSC transplantation and the Rg1 combination treatment

Different from the sham group, reduced Bcl-2 expression and enhanced Bax expression were observed in the IR group (Fig. 6A). Compared with the IR group, Rg1 treatment or the combination treatment significantly increased Bcl-2 protein levels and decreased Bax protein levels (Fig. 6B and 6C). We also calculated the ratio between Bcl-2 and Bax that

directly demonstrated cell apoptosis. As shown in Fig. 6D, Rg1 treatment or the combination treatment significantly enhanced the Bcl-2/Bax ratio, suggesting reduced neuron cell apoptosis.

Discussion

At present, stem-cell based therapy is increasingly attached great importance in regenerative medicine [19]. To reduce the morbidity and mortality of cerebral ischemia reperfusion injury, many scholars began to explore the use of stem cell transplantation to cure the disease, such as BMSC transplantation, human placentas (PMSCs) transplantation [19-21]. After BMSC transplantation, the nerve cell can regenerate and continuously enter into the damaged area. Next, the neural structure can be reconstructed and restored, a finding that can potentially be applied in the prevention and treatment of ischemic encephalopathy [22]. Although BMSC transplantation has been applied in the study of cerebral ischemia disease, it is challenged by a low neural cell conversion rate, weak proliferation ability and short survival time after the transplantation. This result may be related to the microenvironment changes *in vivo*, which greatly affects the therapeutic effect. Thus, identifying a drug that can promote the proliferation of BMSCs and improve the survival rate of BMSCs is a hot research topic. Recent research has shown that atorvastatin helps to improve the microenvironment from two aspects, including synergistically enhancing the effects of BMSCs and adding new therapeutic effects to BMSCs transplantation. The combinational use of atorvastatin and BMSCs transplantation provides a prospective future for the future preclinical and clinical studies in acute myocardial infarction (AMI) [23].

Ginsenoside Rg1 is extracted from ginseng root and stem. As a small molecular substance, it is easy to pass through the blood brain barrier [24, 25]. Ginsenoside Rg1 has anti-inflammatory and antioxidant effects, which can clear free radicals, inhibit protein kinase activation and decrease GSH concentration, thereby providing a good microenvironment for the *in vivo* survival and differentiation of BMSCs [26]. Ginsenoside Rg1 can function as a BMSC-inducing agent, which promotes BMSC differentiation and proliferation [27].

According to our study, significant nervous deficits were found after 24 h of ischemia reperfusion. At 14d, the brain water content was significantly increased. TTC staining demonstrated the larger infarction foci, suggesting successful preparation of the cerebral ischemia model. Compared with the IR group, the symptoms of neurological deficit were markedly alleviated at 7d and 14d post reperfusion. Meanwhile, the brain water content and infarct volume were reduced to different extents, particularly with the combined treatment group, suggesting that each treatment group has obvious therapeutic effects on cerebral ischemia reperfusion injury.

At present, the specific mechanism of cell death after cerebral ischemia is still not clearly known. However, Mitochondria-dependent apoptosis pathways are considered to play key roles in the process, among which the anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax are key regulators in cell survival and cell apoptosis [28]. TUNEL staining demonstrated that the IR group showed obvious ischemic changes and apoptosis. Additionally, in each treatment group, the pathological change was alleviated. The ginsenoside Rg1 treatment significantly decreased the number of apoptotic cells and Bcl-2/Bax ratio. However, after BMSC transplantation, no obvious changes in cell apoptosis were observed. Using immunofluorescence, NSE and GFAP staining were visibly obvious, indicating BMSC differentiation into neurons and glial cells. Additionally, no apoptosis was observed in differentiated BMSC cells. Although apoptosis of the BMSC transplantation group was not significantly changed, the transplanted cells can differentiate into neurons and glial cells to repair damaged brain tissue, so that the ischemic lesion can be improved obviously. Thus, it can be presumed that ginsenoside Rg1 combined with BMSC transplantation can promote brain tissue repair after cerebral ischemia reperfusion injury mainly by enhancing neuron-like cell differentiation and the anti-apoptosis effect of Rg1.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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