Neuroprotective Effects of Bone Marrow Mesenchymal Stem Cells Combined with Ginsenoide Rb1 on Mice with Intracerebral Hemorrhage

Wei Wang¹,²,*, Ling Yin¹,*, Kan Guo¹, Li Deng¹, Xiaqing Gao¹, Jiaxing Pan³, Yan Zhu⁴, Chaoxian Yang⁴

¹Department of Neurobiology, Research Center for Preclinical Medicine, Southwest Medical University, Luzhou 646000, China
²Department of Paediatrics, Center Hospital of Shangqiu, Shangqiu 476000, China
³Department of Clinical Medicine, Southwest Medical University, Luzhou 646000, China
⁴Department of Anatomy, Southwest Medical University, Luzhou 646000, China

Abstract

Aim of the study: To investigate the neuroprotective effects of bone marrow mesenchymal stem cells (BMSCs) combined with ginsenoside Rb1 (GRb1) on mice with intracerebral hemorrhage (ICH).

Methods: C57BL/6 mice with ICH were divided into control group, GRb1 group, BMSCs group and BMSCs + GRb1 group randomly, then each group was subdivided into 14d and 28d subgroups. BMSCs and GRb1 were injected 2 days after ICH. Modified neurological severity scores (mNSS) were used to evaluate the neurological function. Hoechest 33258 staining was used to detect the apoptotic cells and hematoxylin and eosin (H&E) staining was applied to observe the morphological changes of brain tissues. Immunofluorescence was used to assess the differentiation of transplanted BMSCs in vivo.

Results: In four groups, BMSCs+Rb1 group has the lowest mNSS scores and the minimum number of apoptotic cells (p<0.05). Compared with the control group, the degrees of lesion were reduced and the morphological structures were more complete in other three groups. Compared with the BMSCs group, the positive percentages of GFAP (on day 28), MAP2 and nestin (on day 14 and 28) among grafted cells were significantly increased in BMSCs+GRb1 group (p<0.05).

Conclusion: Combined treatment of BMSCs transplantation and GRb1 provide better neuroprotection for mice with ICH.

Keywords: Bone mesenchymal stem cell; Ginsenoside Rb1; Differentiation; Intracerebral hemorrhage

Abbreviations: BMSCs: Bone marrow mesenchymal stem cells, GRb1: Ginsenoside Rb1, RFP: Red fluorescent protein, mNSS: Modified neurological severity scores, H&E: Hematoxylin and eosin, ICH: Intracerebral hemorrhage, GFAP: Glial fibrillary acidic protein, MAP2: Microtubule associated protein 2, SPF: Specific pathogen free, IVC: Individually ventilated cage, FCM: Flow cytometry, PFA: Paraformaldehyde, PBS: Phosphate buffered saline, ANOVA: Analysis of variance, OGD: Oxygen glucose deprivation

Introduction

Intracerebral hemorrhage (ICH) is a devastating subtype of stroke with high morbidity and mortality, and mainly caused by cerebral amyloid angiopathy or hypertension inducing cerebral vascular rupture [1,2]. The hematoma after ICH results in space-occupying effect and direct injury to surrounding tissues. Secondary lesion includes hypoperfusion surrounding the hematoma, cerebral edema, inflammatory reaction, toxicity effect induced by dissolving red blood cells, reactive oxygen species reaction, a chain reaction caused by the generation of prothrombin coagulation, and so forth [3-6]. ICH causes massive neurons death and neurological deficits [7,8], however, regeneration ability of neuron is limited and minimally invasive hematoma aspiration alone could not effectively accelerate the recovery of neurological functions.

At present, medical therapy against ICH shows only limited effectiveness. With the increasing use of stem cells based on cell therapies, bone marrow mesenchymal stem cells (BMSCs) have been gradually becoming the hot spot of the current research. Our previous studies have shown that BMSCs transplantation could decrease modified neurological severity scores (mNSS) and reduce lesion volume in ICH rats [9]. Some studies have reported that BMSCs have the capacity to migrate to the damaged regions and differentiate into neurons and astrocytes improving functional recovery after stroke [10-12], but the differentiation rate of grafted...
BMSCs is not good in vivo, how to regulate and improve the differentiation of transplanted BMSCs for better efficacy is of great importance. Ginsenoside Rb1 (GRb1) has an effective rehabilitation for the central nervous system disease [13,14], but the effect of GRb1 on neural differentiation of transplanted BMSC in mice with ICH has not been investigated. In this study, we examine whether combined treatment of BMSCs and GRb1 offer neuroprotective effects and GRb1 promote neural differentiation of BMSCs in mouse brain with ICH.

**Material and methods**

**Animals**

Specific pathogen free (SPF) male C57BL/6 mice (n=50, 25-35 g) were obtained from the experimental animal center of Third Military Medical University, and raised under individually ventilated cage (IVC) laboratory (temperature 20-22°C, humidity 60-70%) with access to water and food ad libitum. The experimental protocol was in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of the People’s Republic of China.

**BMSCs culture**

BMSCs were isolated and purified from bone marrow, and labeled with red fluorescent protein (RFP), then characterized by flow cytometry (FCM) analysis that included positive assays for anti-CD44-FITC and negative assays for anti-CD44-FITC (Cyagen Biosciences Inc., Guangzhou, China). BMSCs were cultured in an α-MEM medium containing 10% fetal bovine serum in an incubator with 5% CO2 at 37°C. After reaching 80% confluence, the 3rd passage of BMSCs were digested with 0.25% trypsin, then centrifuged and diluted in saline to 5 × 10⁷ cells/ml for cell transplantation.

**ICH model**

ICH was induced by injecting collagenase as previously described [15]. Briefly, C57BL/6 mice were anesthetized with 1% pentobarbital sodium and then placed prostrate in a stereotoxic frame (Angle Two Stereotaxic Instrument, MyNeuroLab, USA). Burr holes (1.5 mm in diameter) were drilled, and 0.075 U/μl collagenase type I (Sigma) in 1 μl saline was injected into the right striatum (coordinates: 2.5 mm lateral, 0.5 mm anterior, and 3.0 mm ventral to the bregma) at a speed of 0.2 μl/min. After placement for 5 minutes, the needle was removed at a speed of 1 mm/min.

**Neurological functional test and experimental groups**

The neurological function of the mouse was evaluated by modified neurological severity scores (mNSS) test as previously described [16]. The neurological function was graded on a scale of 0 to 18 score, and the higher the score is, the heavier the injury is. Only if the score is greater than 8, the mice can be used for the follow-up experiment. ICH mice were divided into 4 groups randomly: control group, GRb1 group, BMSCs group, BMSCs+GRb1 group. Each group then was subdivided into 2 subgroups according to different timepoints day 14 and 28 (n=6, each timepoint).

**Cell transplantation**

Two days after ICH in BMSCs group and BMSCs+GRb1 group, the mice were anesthetized with pentobarbital sodium and placed prostrate in a stereotoxic frame again. 5μl cell suspensions of BMSCs (5 × 10⁷ cells/ml) were injected into the right striatum through the same holes as mentioned above at a speed of 2μl/min. After placement for 5 minutes, the needle was removed at a speed of 1 mm/min.

**GRb1 treatment**

20 mg GRb1 powder (Shanghai source leaf Biological Technology Co., Ltd.) was dissolved in 2ml sterile distilled water, configured to 1 mg/0.1 ml solution. In GRb1 group and BMSCs+GRb1 group, two days after ICH, the mice were administrated with GRb1 solution using the dosage of 50 mg/kg by intraperitoneal injection.

**Cryostat section**

At the end of day 14 and 28, mice were anesthetized deeply with pentobarbital sodium, and perfused by heart with saline and 4% paraformaldehyde (PFA) successively. The brain was sectioned into 2 mm coronal blocks and transferred to 4% PFA solution for 24 hours. After gradient dehydration by 15% and 30% sucrose solution, these tissues were embedded and cut into serial coronal sections (10μm thickness ) with a freezing microtome (Leica, CM 1900).

**Hematoxylin and eosin (H&E) staining**

Frozen sections were stained with haematoxylin and differentiated with 1% hydrochloric acid alcohol, then immersed in ammonia-water and dyed with eosin, followed by dehydrating with gradient ethanol, making transparent in xylene and sealing with resin.

**Hoechst33258 Staining**

Hoechst33258 staining was used to observe apoptosis of the hemorrhagic lesion and the surrounding hemorrhagic area. After being fixed by 4% PFA for 30 minutes and washed with 0.01M phosphate buffered saline (PBS) 3 times for 5 minutes once, frozen sections were stained with hoechst 33258 (Beyotime, China) for 10 minutes. After washing with 0.01M PBS 4 times for 5 minutes once, stained sections were sealed with mounting medium.

**Immunofluorescent staining**

Frozen sections were treated with 4% PFA, 0.3% Triton-X 100, 3% H2O2,10% normal goat serum successively, then incubated with rabbit anti-mouse polyclonal glial fibrillary acidic protein (GFAP) primary antibody (1:100; Sigma) or mouse anti-mouse monoclonal microtubule associated protein 2 (MAP2) antibody (1:100; Santa Cruz) or rabbit anti-mouse polyclonal nestin antibody (1:100; Sigma) overnight at 4 °C, followed by incubating with goat anti-mice or goat anti-rabbit secondary antibody labeled with FITC at 37°C for 30 min. Stained sections were sealed with mounting medium. A negative control was carried out using the same procedures without primary antibody. Fluorescence microscopy with...
laser excitation was conducted as previously described [17]. Immunofluorescence was used to assess the differentiation of transplanted BMSCs in vivo. After the yellow fluorescent cells (merged from green and red fluorescence) and red fluorescent cells (implanted BMSCs in vivo) being counted in 5 non-overlapping surrounding regions (10 × 40) of the transplantation area respectively, the proportion of them is calculated.

**Statistical analysis**
Statistical analyses were performed with SPSS 17.0 for Windows (SPSS Inc., Chicago, USA). Values of all variables were presented as mean ± SE. One-way analysis of variance (ANOVA) was used to determine the effects of different treatments. A $p$-value < 0.05 was considered to indicate a statistically significant difference.

**Results**

**Morphology and Phenotype of BMSCs**
BMSCs labeled with RFP appeared flat, fusiform, polygon and irregular shape, and showed red fluorescence under fluorescence microscope (Figure 1A). The expression of CD117 is negative (2.24%) while CD44 is positive (98.13%) by FCM (Figure 1B, offered by Cyagen Biosciences Inc.). The grafted BMSCs mainly distributed in the surrounding hemorrhagic area in vivo under fluorescence microscope (Figure 1C).

**Neurological Functional Tests**
ICH mouse showed neurological deficit symptoms at 2 hours after operation (Figure 2A) and basal ganglia hemorrhage (Figure 2B) was found at 24 hours after injecting collagenase. According to the mNSS test, neurological function had no apparent difference among 4 groups before treatment. Compared with control group, the scores decreased in other 3 groups on day 14. Especially, the scores were the lowest in BMSCs+GRb1 group on day 14 and 28 ($p < 0.05$; Figure 2C).

**Changes of Morphological structure**
H&E staining revealed that the lateral ventricle enlarged more apparently in the ipsilateral side than that in the contralateral side. At the same timepoint, compared with the control group, the degrees of lesion were reduced and the morphological structures were more complete in other three groups (Figure 3).

**Cell apoptosis**
Hoechst33258 staining showed that the number of apoptotic cells surrounding hemorrhagic area gradually declined with the extension of time in each group. At day 14 and 28, compared with control group, the number of the apoptotic cells reduced in other 3 groups; and it was the lowest in BMSCs+GRb1 group ($p < 0.05$; Figure 4).

**Neural differentiation of BMSCs in vivo**
Immunofluorescence revealed that the transplanted cells characterized by red fluorescence were scattered mainly surrounding the injection site. Compared with BMSCs group, the percentages of GFAP- positive cells (on day 28), MAP2-positive cells and nestin-positive cells (on day 14, 28) were significantly increased in BMSCs+GRb1 group ($p < 0.05$; Figure 5 and 6).
Figure 2: A, Neurological deficit symptoms of C57BL/6 ICH mouse; B, Basal ganglia hemorrhage had been found in C57BL/6 mouse at 24 hours after operation; C, The scores of the Neurological functional test in different groups. * $p < 0.05$, compared with control group; # $p < 0.05$, compared with GRb1 group; ★ $p < 0.05$, compared with BMSCs group.

Figure 3: H&E staining of frozen sections following 14-day treatment. A, Control group; B, GRb1 group; C, BMSCs group; D, BMSCs+GRb group. □ represent partial enlarged view. (Bar = 1 mm)

Figure 4: Hoechst33258 staining showed the apoptotic cells surrounding hemorrhagic area at day 14. A, Control group; B, GRb1 group; C, BMSCs group; D, BMSCs+GRb1 group; E, The number of apoptotic cells. * $p < 0.05$, compared with control group; # $p < 0.05$, compared with GRb1 group; ★ $p < 0.05$, compared with BMSCs group. (Bar = 30 μm)
Plasticity and developmental capacity of stem cells have now been established as a promising tool to restore the degenerative disorders. Some studies have indicated that transplanted BMSCs have the capacity to secrete growth factors and neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor [18-20], and these factors improve local microenvironment which is helpful to neuroregeneration and cell survival, and our data also shows that BMSCs transplantation reduce cell apoptosis in ICH mice. Moreover, BMSC is a kind of seed cells for cell replacement to treat neurological deficits [21,22], but the neural differentiation rate of grafted BMSCs is not good in brain.

Chinese medicinal herbs exhibit certain effects on the treatment of neurological disease [23]. Ginseng root, a well-known traditional Chinese herbal medicine with a long history of usage in East Asian countries, consists of two major ingredients: crude ginseng saponin and crude ginseng non-saponin fractions [24]. GRb1 is a representative substance of crude ginseng saponin.

Experiments showed that GRb1 improved both early and delayed injuries in the thromboembolic stroke model in non-human primates [25]. Qian et al. reported that GRb1 protected neurons against the toxicity of Aβ most through an antioxidant pathway [26]. GRb1 inhibited both oxygen...
glucose deprivation (OGD) and transient ischemia-induced neuronal death and mitigated OGD-induced autophagic vacuoles in SH-SY5Y cells [27]. In this study, we found that GRb1 improved neurobehavioral function and decreased the number of apoptotic cells at day 14 in ICH mice. These studies provided several clues that GRb1 show neuroprotective effects in the central nervous system disease, but the effects of GRb1 on the neural differentiation of transplanted BMSCs in brain is unclear.

In the present study, the positive percentages of GFAP (on day 28), MAP2 and nestin (on day 14 and 28) among grafted cells were increased in BMSCs+GRb1 group ($p<0.05$) when compared with the BMSCs group. These results suggest that GRb1 could promote neural differentiation of transplanted BMSCs in ICH mice. Although GRb1 activates signaling pathways involving PI3K/Akt, Notch, JNK to protect cells [27-29], it is not clear which signaling pathways are involved in GRb1 induced neural differentiation of BMSCs into neuron-like cells. Thus, further studies are warranted to determine these mechanisms.

**Conclusion**

Our research provides evidences that GRb1 could promote neural differentiation of grafted BMSCs and the combined treatment of GRb1 injection and BMSCs transplantation could give advantage to the restoration of neural function on mice with ICH.

**Acknowledgements**

This work is supported by Research Project of Science Foundation of Sichuan Province Educational Commission of China (18ZA0516) and Joint Research Project of Department of Science and Technology of Sichuan Province and Luzhou Municipal People’s Government and Luzhou Medical College (14JC0140).

**References**


Copyright: © 2018 Wang W, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

J Neurol Neuro Toxicol 7 Volume 2(1): 2018