Hypothermia enhances heme oxygenase 1-expressing mesenchymal stem cell-induced repair of cerebral hypoxia-ischemia

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Abstract
Introduction: Mesenchymal stem cells (MSC) are known to exhibit potency for the repair of cerebral hypoxia-ischemia (HI). Heme oxygenase 1 (HO-1) is capable of inducing repair and can boost stem cell-based therapeutic effects. Hypothermia is known to have strong cytoprotective effects.

Material and methods: HO-1 secreting lentiviral vector was constructed and transfected into MSC. HO-1 secretion from HO-1/MSC was verified using Western blot, real-time PCR and ELISA tests. The proliferation and anti-oxidative abilities of HO-1/MSC were detected by CCK-8 assay and biochemical kits. The therapeutic efficacy of HO-1/MSC in a rat cerebral HI model under normothermia or hypothermia was then tested. The cells were transplanted into HI animal models and the rats were kept at 37°C or 25°C. The histological pathology, apoptosis and behavior were analyzed with Nissl stain, TUNEL analysis, and rotarod and cylinder tests respectively. MSC survival and differentiation in vivo were also analyzed by immunofluorescence.

Results: HO-1 secretions promoted the proliferation and anti-oxidative abilities of MSC. The HO-1/MSC significantly improved the apoptosis, injury, behavior and enhanced MSC survival and differentiation into mature neurons and oligodendrocytes. Moreover, HO-1/MSC significantly enhanced repair of HI under hypothermia.

Conclusions: These results suggest that HO-1-secreting MSC under hypothermia is an effective therapeutic approach for the treatment of HI.

Key words: heme oxygenase 1, hypothermia, mesenchymal stem cells, cerebral hypoxia-ischemia.

Introduction

Neonatal cerebral hypoxia-ischemia (HI) is a common cause of death and neurological disability in children and can lead to long-term cognitive delays and behavioral deficits [1]. No effective clinical treatment has been developed so far. Using stem cells or progenitor cells to reduce cerebral impairment or promote cell regeneration is a promising strategy, which has been verified in a variety of functional disorders of the central nervous system and local ischemic diseases [2].

Stem cell transplantation to promote nerve regeneration and improve nerve function is currently a research hotspot [3–5]. To date, various
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Types of stem cell transplantation have been tried in China and abroad for the treatment of HI, including umbilical cord blood derived from embryonic stem cells and bone marrow mesenchymal stem cells (MSC). However, stem cell therapy has encountered various problems, such as low survival and proliferation rates of implanted cells, due to the hostile microenvironment of the infarcted region. Therefore, it is necessary to eliminate the reactive oxygen species (ROS) injury.

Heme oxygenase 1 (HO-1) is an important antioxidant enzyme in the cell, which belongs to stress proteins. It presents adaptive expression in the stressed state, and exerts anti-inflammatory and anti-injury effects. Continuous expression of HO-1 in endothelial cells can reduce the inflammatory damage and apoptosis of cells caused by ischemia [6,7]. It is also found that HO-1 inhibits the formation of ROS, lipid peroxidation, atherosclerosis and inflammation [8,9]. On the other hand, hypothermia is known to have strong cytoprotective effects [10] through many mechanisms, such as lowering the metabolic rate, reducing glutamate release, reducing ROS, modifying inflammation genes and apoptotic protein gene expression. Furthermore, hypothermia treatment in the brain of ischemic neonatal rats could reduce infarct size and dysfunction for a long time [11].

In this study, we developed HO-1-secreting MSC. Then, HO-1/MSC were implanted into a rat cerebral HI model to treat under 37°C or 25°C and to assess whether they can enhance the hypoxia-ischemia protective effect.

Material and methods

Ethics statement

Male 1-week old wild-type SD rats were purchased from Zhongnan Hospital of Wuhan University and were maintained under specific pathogen-free condition. All experiments involving the use of animals were approved by the Committee of Animal Care and Use of Bao’an Maternity and Child Health Hospital, China (BAMCHH-2015121). All animal experiments and procedures were approved by the Institutional Animal Care and Use Committees of Bao’an Maternity and Child Health Hospital, China (BAMCHH-2015121).

Rat mesenchymal stem cells and cell culture

Rat MSC, derived from normal rat bone marrow, were purchased from Cyagen Bioscience, Inc., Guangzhou, China and identified according to the instructions by the supplier. According to the analysis of FACS, the cells can express CD90, CD44, and CD29, but not CD34, CD45, and CD11b/c. The MSC were cultured in rat MSC basal medium supplemented with 10% fetal bovine serum, supplied by Cyagen Bioscience, Inc.

HO-1 secreting lentiviral vector construction

pLV-EGFP-C carrying EGFP was supplied by Inovogen Tech. Inc, Beijing China. Primers were designed according to the HO-1 in GenBank, 5′-TTATGATTTACATTGCACAGCCC-3′ (EcoRI site in bold); and 5′-CGCGGGATCCTTACATGGCTAAATTCCCACTGC-3′ (BamHI site in bold) and synthesized by GenePharma company (Shanghai, China). The PCR product was digested with EcoRI and BamHI and sequenced by ABI377 DNA sequencer (Takara, Shanghai, China). Rat MSC with 10 or so passages were used for transfection. After 80% confluence of MSC in a 6-well plate, the constructed pLV-EGFP: HO-1 or control pLV-EGFP was added into the cells for 24 h. After the old medium was replaced with fresh culture medium, the culture medium was changed every 2–3 days. The transduction efficiency of the lentiviral vectors at 72 h after transfection was identified using fluorescence microscopy (Olympus Co., Tokyo, Japan).

Western blotting

Cells or brain tissues were lysed with RIPA buffer containing protease inhibitors (Dingguo, Beijing, China). Rabbit anti-HO-1 polyclonal antibody (1 : 500), p 65 (1 : 1000), active caspase-3 (1 : 5000), and GAPDH (1 : 10000) were provided by Santa Cruz Biotechnology, CA, USA. A horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1 : 5,000, Boster, Wuhan, China) was used as a secondary antibody [12].

Real-time PCR

RNAs were isolated from MSC and reverse transcribed into cDNA and analyzed by quantitative real-time PCR. Quantitative real-time PCR was performed on an Applied Bio Systems Inc 1900 system using the SYBR Green Real Time PCR Kit (Bio-Rad, America). The primers were synthesized by Hua-da (Shanghai, China). Forty cycles comprising 94°C for 30 s and 60°C for 30 s after an initial 94°C for 4 min were carried out. The fold-change from experimental to control was calculated as $2^{-ΔΔCt}$[13].

ELISA

Heme oxygenase-1 secretion was quantified using an ELISA kit (ab213968, Abcam, USA) according to the instruction supplied by the manufacturer. The catalase (CAT), lactic dehydrogenase (LDH) and superoxide dismutase (SOD) activities were evaluated by kits according to the instructions (Jiancheng, Nanjing, China) [14].
Proliferation assay

The cells were planted into a 96-well plate with 10000 cells per well and incubated at 37°C 5% CO₂ for 12, 24, 48, 72 h, respectively. 10 μl of CCK 8 reagent was added to obtained cells at different time points and incubated for 3 h, then OD 490 nm was recorded and the cell growth curve was plotted. Four independent experiments were performed [15].

Rat models with HI cerebral injury

SD rats aged 1 week were used. Following anesthesia with ketamine (50 mg/kg i.p.), the neck of each rat was cut open along the median line of the abdomen, and then the right common carotid artery was ligated using a surgical thread [16]. After incisions were closed, the rats were kept warm until recovery from anesthesia. Then, the rats were placed in a tightly closed hypoxic chamber for 90 min where a constant hypoxic flow of 8% O₂ and 92% N₂ (350 ml/min) was maintained. The hypoxic chamber was placed in a temperature-controlled water bath to maintain the temperature at 37°C. Following exposure to hypoxia, the offspring were sent to their mothers for recovery. After completion of hypoxic exposure, the juvenile rats were randomized to two groups. The normothermia group and the hypothermia group were placed in chambers in 37°C and 25°C water baths for 24 h, respectively. Animals in the control group were anesthetized but not given HI treatment.

Cell transplantation

Forty-eight rats were randomized to 8 groups as follows (n = 6): control group, HI group, HI + MSC group, HI + HO-1/MSC group under 37°C or 25°C. Following the HI cerebral injury on day 1, each rat was anesthetized (ketamine 50 mg/kg, i.p.) and fixed on a Kopf stereotaxic frame (Kopf, CA, USA), and then 3 μl of MSC (1 × 10⁶ cells/μl) or PBS was injected into the lateral ventricle of the right brain (AP 0.02 mm; ML 0.75 mm; DV 2 mm).

Preparation of brain sections and TUNEL analysis

Four weeks after transplantation, rats were anesthetized with ether, and immediately the rats' brains were removed and fixed in 4% PFA; after being placed in 30% sucrose (w/v) for 3 days, the brains were cut into 15 μm sections using a cryostat. Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) analysis was carried out to identify nucleus DNA fragments in penumbras and ischemic cores. TUNEL staining was performed following the manufacturer's instructions (Calbio, SD, USA), as briefly described below: brain sections were incubated at room temperature with 20 μg/ml proteinase K for 20 min, rinsed with TBS and incubated at room temperature in 1 × TdT enzyme equilibrium buffer for 30 min and then in a TdT labeled reaction mixture at 37°C for 1.5 h; following addition of the stop solution and the blocking buffer, sections were incubated in 1 × conjugate solution at room temperature for 30 min, and TUNEL positive cells were visualized using a DAB kit. Finally, sections were stained with methyl green.

Nissl stain

Four weeks following cerebral hypoxia-ischemia, rat brains were isolated and cut into 6 coronal sections (2 mm thick). The sections were stained with 0.5% cresyl violet acetate. Digital images of all sections were obtained for analysis and evaluation of infarction volumes. The borders between infarction and non-infarction zones were identified using Image J software (NIH). The infarction area was estimated by subtracting the area of the non-infarction area in the ipsilateral hemisphere from the area of the contralateral hemisphere in a blind mode. The infarction volume of the focal zone was calculated by integration [17].

Behavioral tests

A series of behavioral tests were conducted on each rat 4 weeks following transplantation. A rotation test was conducted to assess the degree of hemiplegia and coordinated movement. All rats were trained so that they stayed on the accelerating rotarod, and then a 5 min rotation test was carried out with the rotational speed gradually increased from 4 rpm to 40 rpm. Post-ischemia/hypoxia was expressed as the ratio of the longest duration in three tests to the pre-ischemia/hypoxia baseline value. A cylindrical test was carried out to assess spontaneous movement, especially in case of forelimb inability [18]. Each rat was placed in a transparent acrylic cylinder 6 cm in diameter and 10 cm in height. A mirror was placed behind the cylinder at an appropriate angle and their activity was videotaped for 5 min. The number of wall contacts with forelimbs was counted. The wall contacts were classified as contact by contralateral forelimb (CF), ipsilateral forelimb (IF), or both forelimbs (BF). The percentage of the uses of CF and BF relative to the total number of contacts was calculated as follows: total percentage = (CF + BF/2)/(CF + BF + IF) × 100.

Immunofluorescence and cell counting

Brains of rats were removed and cut into 5 μm coronal sections as mentioned above. Prior to immunostaining, the floating parts and cell cultures were permeated for 5 to 10 min in PBS buffer con...
Figure 1. HO-1-secreting MSC construction. The transduction efficiency was assayed by fluorescence microscopy (A) **p < 0.01 vs. MSC. MSC – empty vector-transfected MSC.
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**Results**

**HO-1-secreting MSC construction**

The successful transfection was detected by fluorescence microscopy for the expression of GFP. As shown in Figure 1 A, after transfection of a lentiviral vector with a multiplicity of infection (MOI) of 100 for 72 h, the transduction efficiency was above 90%. Next, the HO-1 expression was verified by Western blotting and real-time PCR. HO-1 expression was significantly induced in the cell lysates after lentivirus infection (Figures 1 B, C).

Moreover, we found the induction of the HO-1 secretion into the media using ELISA (Figure 1 D). From our results, it was confirmed that HO-1/MSC secreted HO-1 into the cell culture media.

**Increased anti-oxidation and proliferation of HO-1-secreting MSC**

To examine the biological effects of HO-1 secreted from HO-1/MSC, the expression levels of SOD, CAT and LDH in each group were identified with biochemical kits. The HO-1 secreting MSC showed a significant increase in the activities of SOD and CAT and a reduction in the activities of LDH (Figures 2 A–C). These results indicated that the HO-1 secreting MSC exhibited strong anti-oxidation effects. Moreover, the results of the CCK-8 assay showed that the HO-1 secreting MSC had stronger proliferative abilities (Figure 2 D).

**Protective effects of cell transplantation into rats**

Rats exhibited marked histological changes 2 days, 1 week and 2 weeks following HI (Figure 3 A). Unknown cerebral infarctions were found in the

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**Statistical analysis**

All statistical analyses were performed with SPSS 15.0 to assess differences among the groups. The Mann-Whitney U test was performed as a post hoc test. A p-value less than 0.05 was considered to be statistically significant.
right brain 2 days after HI. Additionally, the lesioned part liquefied after 1 week. Finally, the liquefied zone became a cyst after 2 weeks, which, as shown in Figure 3 A, was located 0.92 mm from the posterior fontanelle on the coronal section of the brain. The dashed square denotes the area used for evaluating positive cells.

Four weeks following cell transplantation, cerebral infarction volumes of rats having received cell transplantation were in the following order: HI > MSC > HO-1/MSC; in the hypothermia group, the same rule was found, but infarction volumes were significantly smaller than in the normothermia group (Figure 3 B).

Apoptosis was assayed by TUNEL staining. The results showed that, 4 weeks following cell transplantation, TUNEL positive cells were in the following order: HI > MSC > HO-1/MSC group. In the hypothermia group, the same rule was found, and TUNEL positive cell reductions were more pronounced (Figure 3 C).

As shown in Figure 3 D, NF-κB and active caspase-3 levels in the HI group were significantly higher than those in the control. Under normothermia, compared with the HI group, NF-κB and active caspase-3 in the HO-1/MSC group decreased significantly, while no significant difference was observed in the MSC group. In the hypothermia group, the same rule was found, but NF-κB and active caspase-3 levels were lower than those under normothermia.

Effects of cell transplantation on functional recovery

In the rotation test, behaviors of rats significantly improved from day 14 in the HI group under hypothermia when compared with those in the HI group under normothermia. Moreover, behaviors of rats most significantly improved in the HO-1/MSC group under hypothermia from day 14 after cell transplantation (Figure 4 A).
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Figure 3. Protective effects of HO-1-secreting MSC on HI was assayed by histological changes (A), TUNEL stain (B, n = 6), Nissl stain (C, n = 6) and western blot for p65 and caspase-3 (D, n = 6) 4 weeks following cell transplantation. *p < 0.05 and **p < 0.01 vs. respective HI.

Survival and differentiation of transplanted cells
Labeled with GFR stem cells appeared in green under a fluorescence microscope. The number of transplanted cells in the right cerebral cortex decreased, functional recovery was better in the HI group under hypothermia from day 7 than in the HI group under normothermia and HO-1/MSC under hypothermia showed a significant difference with the HI group under hypothermia from day 28 following cell transplantation (Figure 4 B).
creased over time. We blindly counted cortical cells under a microscope (400×). Four weeks following transplantation, the number of GFP positive cells was significantly greater in the HO-1 + MSC group than in the MSC group, and hypothermia favored survival of transplanted cells. Stem cells differentiated into Iba1 (marker of oligodendrocytes), NeuN (marker of mature neurons) and GFAP (marker of astrocytes) positive cells in HO-1+MSC groups are shown in Figure 5 A. Four weeks following transplantation, HO-1 + MSC was more facilitative than MSC for differentiation into NeuN positive and Iba1 positive cells, and hypothermia was helpful for the differentiation of transplanted cells (Figure 5 B).

Discussion

Stem cell-based therapy has been widely investigated for the treatment of HI. In recent studies, stem cell therapy using MSC offered a positive result for the treatment of HI [19, 20]. However, the application of cell therapy faces many obstacles due to the low survival of the implanted cells and insufficient oxygen and nutrients. Moreover, there are a number of studies utilizing MSC to treat HI, but therapeutic limits existed in these studies. To increase the therapeutic potential of MSC, it requires combination with other protective factors such as hypothermia [21] or HO-1. To overcome these obstacles with ischemic disease, we generated HO-1-secreting MSC and examined their therapeutic effects under hypothermia.

In this study, in vitro, we found that HO-1 enhanced the proliferation and anti-oxidation of MSC. Moreover, MSC/HO-1 showed more significant improvements in the volume of focal cerebral ischemic infarction, apoptosis, behaviors, the survival and differentiation of transplanted cells in vivo as compared to therapy with MSC. The impaired cerebral tissue may have been regenerated better by the transplanted stem cells in some reports [22]. However, the cerebral regenerative effect was not clearly observed in our MSC alone group, and other researchers also reported that the frequency of the occurrence of these regenerative processes by MSC was very low. However, our combined approaches of induced HO-1 greatly contributed to the prevention of cerebral hypoxia-ischemia. The apparent improvement observed for this combination therapy can be explained as follows: First, one therapeutic effect might come from HO-1. Our data showed that the induced HO-1 could enhance the proliferation and anti-oxidation, and finally prevent cerebral HI by improving the behaviors of HI rats, the implanted cell survival and differentiation in the badly infarcted region. Furthermore, the increased antioxidation may contribute to protection against cerebral HI. Second, some studies have proved that hypothermia can reduce the volume of cerebral ischemic infarction. It has a strong neuroprotective effect through many known mechanisms, such as reducing metabolic rate and glutamate release, thereby reducing ROS and preventing destruction of the blood-brain barrier [23]. In our study, hypothermia alone caused a significant improvement of the volume of cerebral ischemic infarction and apoptosis, the behaviors of HI rats and the implanted cell survival and differentiation. HO-1 and hypothermia are synergetic.

In conclusion, we have successfully developed rat HO-1-secreting MSC and the cells significantly improved the cerebral hypoxia-ischemia under hypothermia. Therefore, HO-1-secreting MSC transplantation provides an effective treatment for HI.

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Figure 5. Survival and differentiation of transplanted cells 4 weeks following cell transplantation (n = 6). A – representative images in HO-1/MSC group.
analysis, and interpretation of data, or in writing the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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