Preconditioning of bone marrow mesenchymal stem cells with hydrogen sulfide improves their therapeutic potential

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Keywords: hydrogen sulfide, bone marrow mesenchymal stem cells, transplants, brain-derived neurotrophic factor, vascular endothelial growth factor

Received: February 08, 2016    Accepted: July 27, 2016    Published: August 09, 2016

ABSTRACT

Bone marrow mesenchymal stem cells (BMSCs) transplantation has shown great promises for treating various brain diseases. However, poor viability of transplanted BMSCs in injured brain has limited the therapeutic efficiency. Hypoxia-ischemic injury is one of major mechanisms underlying the survival of transplanted BMSCs. We investigated the mechanism of preconditioning of BMSCs with hydrogen sulfide (H2S), which has been proposed as a novel therapeutic strategy for hypoxia-ischemic injury. In this study, we demonstrated that preconditioning of NaHS, a H2S donor, effectively suppressed hypoxia-ischemic-induced apoptosis whereby the rise in Bax/Bcl-2 ratio. Further analyses revealed Akt and ERK1/2 pathways were involved in the protective effects of NaHS. In addition, NaHS preconditioning increased secretion of BDNF and VEGF in BMSCs. Consistent with in vitro data, transplantation of NaHS preconditioned BMSCs in vivo further enhanced the therapeutic effects of BMSCs on neuronal injury and neurological recovery, associated with increased vessel density and upregulation of BDNF and VEGF in the ischemic tissue. These findings suggest that H2S could enhance the therapeutic effects of BMSCs. The underlying mechanisms might be due to enhanced capacity of BMSCs and upregulation of protective cytokines in the hypoxia tissue.

INTRODUCTION

A growing number of experimental studies highlights the potential of stem cell transplantation as a novel therapeutic approach for ischemic stroke [1]. Moreover, a variety of clinical trials have been performed and others are currently ongoing [2]. Bone marrow-derived mesenchymal stem cells (BMSCs) are widely used in cell therapy because of their ease of acquisition and expansion, immune tolerance, and differentiation capacity [3, 4]. Transplantation of BMSCs in the acute stage of ischemic stroke often modulates the inflammatory milieu, reduces lesion size and inhibits apoptosis in the penumbra area by providing neuroprotective paracrine factors [3, 5, 6]. However, only a small fraction of grafted cells survived in the ischemic brain 28 d after grafting because of the interplay of poor blood supply, ischemia reperfusion, inflammation, and apoptosis. Donor variability is another challenge for BMSC therapy trials.

Hydrogen sulfide (H₂S) has been classified as a novel gasotransmitter signaling molecule in the central nervous system (CNS), involving in the regulation of ion channels, neurotransmitter functions, and other intracellular signaling molecules such as tyrosine kinases [7]. Interestingly, accumulating evidence has been garnered which suggests that exogenous H₂S can function as a powerful neuroprotective agent. Kimura et al. demonstrated that H₂S protected primary rat cortical neurons from oxidative stress-induced injury [8]. H₂S exerts a number of cytoprotective and anti-inflammatory, anti-oxidant, and anti-apoptotic
RESULTS

H2S preconditioning promotes proliferation of BMSCs under hypoxia-ischemic condition

The effects of various concentrations of NaHS (0.1, 0.5, 1, 5, 10 and 50 μM) on viability of BMSCs were assessed. MTT results showed that NaHS preconditioned BMSCs (PC-BMSCs) had a significant reduction in death, compared with the non-preconditioned BMSCs (non-PC-BMSCs) after 48 h (p < 0.01) and 72 h (p < 0.01) hypoxia-ischemic injury. Furthermore, 1 μM NaHS preconditioning yielded the optimal effect on cell viability (82.8 ± 6.07 % vs 64.2 ± 7.77 % at 48 h, p < 0.01; and 108.9 ± 9.12 % vs 44.0 ± 5.24 % at 72 h, p < 0.01, respectively) (Figure 1A). Given the effectiveness of NaHS preconditioning at these concentrations (0.1, 1 and 5 μM), this protocol was used in the most of the subsequent in vitro experiments, unless otherwise stated. In addition, cell viability following treatment with NaHS alone at 0.1 μM (99.9 ± 8.64%), 1 μM (98.3 ± 9.79%) and 5 μM (104.6 ± 11.38%) was not significantly different from the control group (100 ± 9.75%) at 72 h. To further confirm this result, the number of colonies was counted by crystal violet staining (Figure 1B and 1C). The result indicated that the number of MSCs preconditioned with NaHS (1 μM) increased faster than that of hypoxia-ischemic exposure cells.

We then took BrdU staining assay to quantify the proliferating BMSCs. The result showed that the ratio of BrdU positive cells against total BMSCs were significantly decreased under the 72 h hypoxia-ischemic condition (p < 0.001), while the effects were significantly attenuated by co-treated with NaHS (1 μM) (Figure 1B and 1D, p < 0.01).

H2S preconditioning suppresses apoptosis of BMSCs under hypoxia-ischemic condition

The TUNEL assay revealed that TUNEL-positive BMCSe were significantly increased under the 72 h hypoxia-ischemic condition (p < 0.001), while the effect of hypoxia-ischemic on apoptosis of cells was significantly attenuated by co-treated with NaHS (1 μM) (Figure 1B and 1E, p < 0.01).

Depolarization of the inner MMP is a sign of apoptosis [16]. Therefore, in order to ascertain whether NaHS preserves mitochondrial integrity through the maintenance of MMP, we performed JC-1 staining. As shown in Figure 3B, the red/green ratio of JC-1 was decreased in the BMSCs exposed to hypoxia-ischemic insult compared with the normal group, and this effect was reversed by NaHS (1 μM), which is consistent with the TUNEL assay (Figure 1B and 1F).

H2S preconditioning decreased Bax/Bcl-2 ratio in BMSCs under hypoxia-ischemic condition

Because changes in expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 control the mitochondrial pathway of apoptosis, we examined the expression of Bax and Bcl-2 at the proteins and mRNA levels. As shown in Figure 2A and 2B, hypoxia-ischemic exposure markedly increased Bax, decreased Bcl-2, thereby increased the ratio of Bax/Bcl-2 at both protein and mRNA levels at 72 h post-injury (p < 0.001, p < 0.001, respectively). However, this signal increase was reduced by preconditioning with 1 μM NaHS (p < 0.001, p < 0.001, respectively). In addition, NaHS (1 μM) treatment alone did not significantly alter the Bax/Bcl-2 ratio.

H2S preconditioning did not affect caspase-3 activation in BMSCs under hypoxia-ischemic condition

To further investigate the potential protective mechanism of NaHS, we performed Western blot to assess cleaved-caspase-3 protein levels after hypoxia-ischemic insult. As shown in Figure 2C, hypoxia-ischemic exposure significantly increased cleaved-caspase-3 protein levels (p < 0.01) compared with the corresponding controls. However, preconditioning with NaHS did not affect this hypoxia-ischemic induced cleaved-caspase-3 increase (p > 0.05).

ERK1/2 and Akt pathway mediates the protection of H2S preconditioning in BMSCs under hypoxia-ischemic condition

The roles of ERK1/2 and Akt pathways upon the neuroprotective effects of H2S were assessed using Western blot analysis. As shown in Figure 3A, the levels of phosphorylation of ERK1/2 and Akt were decreased when exposed to hypoxia-ischemic insult. Preconditioning with 1 μM NaHS for 60, 120 and 240 min were all effective in up-regulating ERK1/2 activation in the hypoxia-ischemic group (Figure 3A). To implicate ERK1/2 in NaHS-induced
protection against injury, BMSCs were treated with the specific blocker PD98059 to inhibit this pathway. PD98059 (5 μM) reversed the effect of NaHS on ERK1/2 activation in the hypoxia-ischemic group (Figure 3B). Moreover, analysis by MTT showed that PD98059 (5 μM) attenuated the anti-apoptotic effect of NaHS preconditioning after 72 h hypoxia-ischemic exposure (Figure 3C). In parallel with its effect on phosphorylation of ERK1/2, NaHS preconditioning for 5, 15 and 30 min caused a significant increase in Akt phosphorylation (Figure 3A). Administration of the potent PI3K inhibitor, wortmannin (1 μM), markedly reversed the effect of NaHS on Akt phosphorylation (Figure 3B), and blocked the anti-apoptotic actions of NaHS after 72 h hypoxia-ischemic exposure (Figure 3C).

H2S preconditioning promotes BDNF and VEGF release from BMSCs

BMSCs have been shown to benefit neurological recovery after cerebral ischemia. The possible mechanisms are excreting trophic factors. Thus, we further investigated the effect of preconditioning BMSCs with NaHS (1 μM) on neuroprotective factors such as BDNF and VEGF.

![Figure 1](image-url) **Figure 1: Effects of NaHS treatment on cell viability in bone marrow mesenchymal stem cells (BMSCs) *in vitro*.**

(A) BMSCs maintained in 1% serum medium exposed to hypoxia (hypoxia-ischemic injury) were incubated in the absence or presence of indicated concentrations of NaHS (0.1–50 μM) for 48 h and 72 h and cell viability was examined by MTT assay. Values of cell viability were expressed as a percentage relative to those obtained in controls. Values represent the mean ± SD of n = 6. (B) BMSCs exposed to hypoxia-ischemic were incubated in the absence or presence of indicated concentrations of NaHS (1 μM) for 72 h. The cells were then subjected to crystal violet assay, BrdU assay (red), tunnel staining, and JC-1 staining, counterstained with DAPI (blue). Scale bar = 50 μm. (C) The graphs indicate the number of positive colonies/well by crystal violet staining, n = 6. (D) Quantification of BrdU positive BMSCs over the total DAPI-positive cells, n = 4. (E) Quantification of Tunnel positive BMSCs over the total DAPI-positive cells. Values represent the mean ± SD of n = 4. (F) Quantitative analysis of the ratio of red/green fluorescence, n = 6. Values represent the mean ± SD. **p < 0.01, ***p < 0.001 Hypoxia (Hy) VS Control (Con); #p < 0.05, ##p < 0.01 Hy + NaHS VS Hy.
As shown in Figure 4A, NaHS treatment promoted RNA and proteins expression of BDNF and VEGF of BMSCs both in normal and hypoxia-ischemic conditions, compared with no NaHS treatment. Exposure to hypoxia-ischemic insult slightly decreased VEGF mRNA in comparison to control group while the differences were not statistically significant (Figure 4A, \( p > 0.05 \)).

**H\(_2\)S preconditioning inhibits hypoxia-induced HIF-1α expression in BMSCs**

As shown in Figure 4B, hypoxia-ischemic exposure markedly increased HIF-1α at both protein and mRNA levels at 72 h post-injury (\( p < 0.01 \), \( p < 0.01 \), respectively). However, this signal increase was reduced by preconditioning with 1 μM NaHS (\( p < 0.05 \), \( p < 0.01 \), respectively). In addition, NaHS (1 μM) treatment alone did not significantly alter the HIF-1α level.

**Administration of \( \text{PCBMS} \)Cs improves a greater neurological function in vivo**

To investigate the effects of \( \text{PCBM} \)SCs in protecting neurons from hypoxia-ischemia injury *in vivo*, we established ischemic stroke models that were induced by MCAO in rats, and then transplanted \( \text{PCBM} \)SCs into these rats intravenously. In the beginning, we evaluated neurological function using mNSS. The results showed...
Figure 3: Effect of NaHS on ERK and Akt phosphorylation in BMSCs in vitro. (A) BMSCs exposed to hypoxia-ischemic were incubated in the absence or presence of NaHS (1 μM) for indicated times and total protein was subjected to Western blot analysis. Bar graphs showing quantification of expression levels of phosphor-ERK/ERK or phosphor-Akt/Akt was determined by the Image-Pro Plus 6.0. \( n = 3 \). (B) BMSCs exposed to hypoxia-ischemic were incubated in the absence or presence of NaHS (1 μM), PD98059 (PD, 5 μM) or wortmannin (1 μM) for indicated times and total protein was subjected to Western blot analysis. Bar graphs showing quantification of expression levels of phosphor-ERK/ERK or phosphor-Akt/Akt was determined by the Image-Pro Plus 6.0. \( n = 3 \). (C) BMSCs were pretreated with PD98059 (PD, 5 μM) and wortmannin (1 μM) for 30 min, and then exposed to hypoxia-ischemic in the absence or presence of NaHS (1 μM) for 72 h. And cell viability was examined by MTT assay. Values of cell viability were expressed as a percentage relative to those obtained in controls, \( n = 6 \). Values represent the mean ± SD. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) Hy VS Con; # \( p < 0.05 \), ## \( p < 0.01 \) Hy+ NaHS VS Hy; & \( p < 0.05 \), && \( p < 0.01 \) Hy+ PD/ HY + wortmannin VS HY.
there was no difference in mNSS among the no BMSCs group of rats (MACO group), non-PCBMSCs group and PCBMSCs groups at day 1 \[F (2, 17) = 0.166, p > 0.05\] and day 3 \[F (2, 17) = 1.099, p > 0.05\] after the transplantation. However, neurological deficits were improved in all groups at day 14 after transplantation \[F (2, 17) = 20.043, p < 0.001\]. Scores in non-PCBMSCs group were lower than those in the sham control \(p < 0.05\). When compared with the non-PCBMSCs group, transplantation with PCBMSCs further reduced the scores \(p < 0.05\) (Figure 5A).

**Administration of PCBMSCs reduces infarct volume following ischemic insults in vivo**

The pale stained area was determined to indicate the infarct area (Figure 5B and 5C). The infarct volume in the MACO group (26.1% ± 1.72%) was significantly larger than that in the non-PCBMSCs group (19.1% ± 2.76%, \(p < 0.05\)) at day 14 after transplantation. When compared with the non-PCBMSCs group, transplantation with PCBMSCs (12.2% ± 2.08%, \(p < 0.05\)) further reduced the infarct volume.

**Figure 4: NaHS stimulated the release of BDNF and VEGF, and HIF-1a expression in BMSCs in vitro.** (A) BMSCs exposed to hypoxia-ischemic were incubated in the absence or presence of NaHS (1 μM) for 72 h. The mRNA and protein levels of BDNF, VEGF, and β-actin were then analyzed by western blot and semiquantitative RT-PCR. (B) BMSCs exposed to hypoxia-ischemic were incubated in the absence or presence of NaHS (1 μM) for 72 h. The mRNA and protein levels of HIF-1α and β-actin were then analyzed by western blot and semiquantitative RT-PCR. Each value was normalized to β-actin. Data was expressed as the mean ± SD of \(n = 4\). *\(p < 0.05\), **\(p < 0.01\) NaHS VS Con; #\(p < 0.05\), ##\(p < 0.01\) HY + NaHS VS HY.
**PCBMSCs transplantation reduces neuronal injury in vivo**

Next we examined the cell morphology by HE staining. In the MACO group, cells were arranged sparsely and the cell outline was fuzzy in the infarct zone. Moreover, obvious edema was found in the infarct zone, and more necrotic cells were found in MACO group, while non-PCBMSCs and PCBMSCs transplantation alleviated edema and these morphological damages (Figure 5D). Importantly, the amelioration of cellular morphology was more significant in PCBMSCs group than that in non-PCBMSCs group. As shown in Figure 5D, the cell contour was distinguishable and nucleoli in the center of cells were observed clearly in PCBMSCs group, and only a few neurons were shown to have nuclear pyknosis, hyperchromasia and extremely loose organization.

As shown in Figure 5D, MAP-2 immunolabeling was markedly diminished in the infarct zone, and there was an abrupt transition between the infarct and adjacent normal tissue with occasional MAP-2 positive cells in the transition zone in all groups. MAP-2 positive cells in the non-PCBMSCs and PCBMSCs transplantation group were more than those in the MCAO group. Moreover, MAP-2 positive cells in the PCBMSCs group were significantly higher than those in the non-PCBMSCs groups and (Figure 5D).

**Effect of PCBMSCs on expression of CD31 in cerebral ischemia tissue after cerebral infarction**

CD31 is mainly distributed in vascular endothelial cells and it is usually used to evaluate the vessel density in the tissue. As shown in Figure 5D and 5E, compared with the MACO group, the number of CD31-positive cells was increased in the non-PCBMSCs group and PCBMSCs (p < 0.01, p < 0.05, respectively) at day 14 after transplantation. The number of CD31-positive cells in the PCBMSCs group was significantly higher than that of the non-PCBMSCs (p < 0.05, respectively) (Figure 5E).

**PCBMSCs induces anti-apoptotic effect on neuronal cells in vivo**

Ischemia induced significant TUNEL-positive cells in the ischemic penumbra at day 14 after MCAO (Figure 6A). Quantitative analysis demonstrated that the number of TUNEL-positive cells in the non-PCBMSCs and PCBMSCs group was lower than that in the MACO group (20.2% ± 2.67 vs 33.2% ± 5.39%, p < 0.001; 10.3 ± 2.90 vs 33.2% ± 5.39%, p < 0.001, respectively). Of note, the number of TUNEL-positive cells in the PCBMSCs group was significantly lower than that of the non-PCBMSCs groups (10.3 ± 2.90 vs 20.2 ± 2.67%, p < 0.05) (Figure 6A).

**PCBMSCs decreases Bax/Bcl-2 ratio in vivo**

The results demonstrated that compared with the MACO group, the ratio of Bax/Bcl-2 protein was decreased in the non-PCBMSCs group (p < 0.01). The ratio of Bax/Bcl-2 protein in the PCBMSCs group was significantly lower than that of non-PCBMSCs groups (p < 0.05) (Figure 6B) at day 3 after transplantation. And there was no significantly difference between groups at day 14 after transplantation (p > 0.05) (Figure 6B). Similarly, the ratio of Bax/Bcl-2 mRNA was lower in the non-PCBMSCs group when compared with that of the MACO group at day 3 and 14 after transplantation (p < 0.001, p < 0.001, respectively). The ratio of Bax/Bcl-2 mRNA in the PCBMSCs group was significantly lower than that of the non-PCBMSCs (p < 0.01, p < 0.05, respectively) (Figure 6B).

**PCBMSCs improves BDNF and VEGF expression in vivo**

The levels of BDNF protein in the non-PCBMSCs group were significantly increased at day 3 and 14 after transplantation when compared with the MACO group (p < 0.05, p < 0.01, respectively) (Figure 6C). Moreover, transplanted with NaHS preconditioned BMSCs further upregulated expression of BDNF protein at day 3 after transplantation (p < 0.01) (Figure 6C). In addition, the levels of VEGF protein in the non-PCBMSCs group were significantly increased at day 14 after transplantation when compared with the MACO group (p < 0.01). Transplanted with NaHS preconditioned BMSCs further upregulated expression of VEGF protein at day 3 after transplantation (p < 0.01), when compared with the non-PCBMSCs group (Figure 6C).

As shown in (Figure 6C), non-PCBMSCs treatment significantly increased mRNA levels of BDNF at day 3 and 14 after transplantation (p < 0.01, p < 0.01, respectively) and VEGF (p < 0.001, p < 0.001, respectively), as compared to the MACO group. PCBMSCs groups treatment caused a further increase in the mRNA levels of BDNF at day 3 and 14 after transplantation (p < 0.01, p < 0.05, respectively) and VEGF (p < 0.01, p < 0.01, respectively), when compared to the non-PCBMSCs group.

**DISCUSSION**

We have shown in this study that H$_2$S preconditioned BMCSs not only have a better survival rate than controls, but also express higher levels of BDNF and VEGF, resulting in stronger therapeutic effects in ischemic stroke. The major findings are: (a) H$_2$S protected BMCSs from hypoxia-ischemic injury via down-regulation of Bax/Bcl-2 ratio in vitro and in vivo, (b) H$_2$S preconditioning increased the BMSCs to release BDNF and VEGF in vitro and in vivo, (c) H$_2$S increased the proliferation capacity of the BMCSs, via Akt and ERK1/2 pathways in vitro,

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**PCBMSCs transplantation reduces neuronal injury in vivo**

The cerebral ischemia tissue after cerebral infarction (Figure 6A). Quantitative analysis demonstrated that the number of TUNEL-positive cells in the non-PCBMSCs and PCBMSCs transplantation group was lower than that in the MACO group (20.2% ± 2.67 vs 33.2% ± 5.39%, p < 0.001; 10.3 ± 2.90 vs 33.2% ± 5.39%, p < 0.001, respectively). Of note, the number of TUNEL-positive cells in the PCBMSCs group was significantly lower than that of the non-PCBMSCs groups (10.3 ± 2.90 vs 20.2 ± 2.67%, p < 0.05) (Figure 6A).

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The results demonstrated that compared with the MACO group, the ratio of Bax/Bcl-2 protein was decreased in the non-PCBMSCs group (p < 0.01). The ratio of Bax/Bcl-2 protein in the PCBMSCs group was significantly lower than that of non-PCBMSCs groups (p < 0.05) (Figure 6B) at day 3 after transplantation. And there was no significantly difference between groups at day 14 after transplantation (p > 0.05) (Figure 6B). Similarly, the ratio of Bax/Bcl-2 mRNA was lower in the non-PCBMSCs group when compared with that of the MACO group at day 3 and 14 after transplantation (p < 0.001, p < 0.001, respectively). The ratio of Bax/Bcl-2 mRNA in the PCBMSCs group was significantly lower than that of the non-PCBMSCs (p < 0.01, p < 0.05, respectively) (Figure 6B).

**PCBMSCs improves BDNF and VEGF expression in vivo**

The levels of BDNF protein in the non-PCBMSCs group were significantly increased at day 3 and 14 after transplantation when compared with the MACO group (p < 0.05, p < 0.01, respectively) (Figure 6C). Moreover, transplanted with NaHS preconditioned BMCSs further upregulated expression of BDNF protein at day 3 after transplantation (p < 0.01) (Figure 6C). In addition, the levels of VEGF protein in the non-PCBMSCs group were significantly increased at day 14 after transplantation when compared with the MACO group (p < 0.01). Transplanted with NaHS preconditioned BMCSs further upregulated expression of VEGF protein at day 3 after transplantation (p < 0.01), when compared with the non-PCBMSCs group (Figure 6C).

As shown in (Figure 6C), non-PCBMSCs treatment significantly increased mRNA levels of BDNF at day 3 and 14 after transplantation (p < 0.01, p < 0.01, respectively) and VEGF (p < 0.001, p < 0.001, respectively), as compared to the MACO group. PCBMSCs groups treatment caused a further increase in the mRNA levels of BDNF at day 3 and 14 after transplantation (p < 0.01, p < 0.05, respectively) and VEGF (p < 0.01, p < 0.01, respectively), when compared to the non-PCBMSCs group.

**DISCUSSION**

We have shown in this study that H$_2$S preconditioned BMCSs not only have a better survival rate than controls, but also express higher levels of BDNF and VEGF, resulting in stronger therapeutic effects in ischemic stroke. The major findings are: (a) H$_2$S protected BMCSs from hypoxia-ischemic injury via down-regulation of Bax/Bcl-2 ratio in vitro and in vivo, (b) H$_2$S preconditioning increased the BMCSs to release BDNF and VEGF in vitro and in vivo, (c) H$_2$S increased the proliferation capacity of the BMCSs, via Akt and ERK1/2 pathways in vitro,
(d) transplantation of the H\textsubscript{2}S preconditioned BMCSs attenuated infarct size and improved neurological recovery in MACO rats.

The mechanisms responsible for the cytoprotective benefits of H\textsubscript{2}S are not yet fully elucidated, but a number of suggestions have been provided. There are data which indicate that H\textsubscript{2}S exerts its cytoprotective benefits against excitotoxic insult as demonstrated both \textit{in vitro} [17, 18] or \textit{in vivo} in neurons [19, 20], and H\textsubscript{2}S also reduces cytotoxicity in astrocytes and microglia [21]. H\textsubscript{2}S exerts its anti-apoptotic effects through various mechanisms, including inhibition of cytochrome c release from mitochondria, normalized Bax /Bcl-2 levels, suppression of caspase-9/3 activation and poly(ADP-ribose) polymerase cleavage [7]. In this study, H\textsubscript{2}S preconditioning reduced apoptosis in the BMSCs and resulted in down-regulation of Bax/Bcl-2 ratio after hypoxia-ischemic insult.

Akt and ERK pathways are involved in numerous crucial cell functions, such as proliferation, differentiation, motility, survival, and intracellular trafficking [22, 23]. And previous studies have shown that H\textsubscript{2}S possesses neuroprotective effects in various models, some of which were dependent on the activity of the ERK and PI3K/Akt

Figure 5: Administration of NaHS preconditioned-BMSCs (PCBMSCs) improved neurological function, decreased infarct volume, promoted neuronal density and MAP-2-positive cells in the infarct area. (A) The scores of mNSS were measured at day 1, 3 and 14 after BMSCs transplantation in MACO group, \textsuperscript{≈}PCBMSCs group and \textsuperscript{\textsuperscript{≠}PCBMSCs group.} \(n = 6\). (B) Brain sections stained with TTC to visualize the ischemic lesions at day 14 after BMSCs transplantation in MACO group, \textsuperscript{≈}PCBMSCs group and \textsuperscript{\textsuperscript{≠}PCBMSCs group.} (C) Quantitative analysis of the Infarct Volume. \(n = 3\). (D) Representative photomicrographs (×10) of the infarct area by HE staining. Scale bar = 100 μm. \(n = 4\). And Representative photomicrographs (×20) of MAP-2 immunohistochemistry in the infarct area. Scale bar = 50 μm. \(n = 4\). (E) Representative photomicrographs of CD31 immunohistochemistry in the infarct area. Scale bar = 100 μm. (F) Quantitative analysis of CD31 positive cells. \(n = 4\). Values represent the mean ± SD, *\(p < 0.05\), **\(p < 0.05\) \textsuperscript{≈}PCBMSCs VS MACO; #\(p < 0.05\) \textsuperscript{\textsuperscript{≠}PCBMSCs VS \textsuperscript{≈}PCBMSCs.
signaling pathway. For example, H₂S therapy significantly suppressed caspase-3 activation and decreased myocardial injury via activating the ERK signaling pathway [24]. Furthermore, by activating the PI3K/Akt signaling pathway, H₂S can increase Bcl-2 protein levels, inhibit activation of the cytochrome c-caspase-3/9 apoptosis pathway, and promote the survival of cells [25]. In line with these findings, our data indicate that H₂S administration significantly enhanced Akt and ERK phosphorylation, and Akt or ERK inhibitor attenuated NaHS’s promotion effects of cell survival, indicating that NaHS exerts its protective property in BMCSs after hypoxia-ischemic insult, at least in part, through the activation of Akt and ERK pathway.

Previous studies showed that intravenous administration of BMSCs after global cerebral ischemia decreased neural damage, suggesting that BMSCs increase neuron survival by intravenous infusion [6, 26, 27]. In this study, we found that BMSCs attenuated infarct size and improved the neurological deficits from day 14 after ischemic stroke and transplantation onward, indicating that neuroprotective effect is one of the main mechanisms of stem cell action in the present study. The paracrine factors produced by BMSCs such as VEGF [28], BDNF [29], and NGF [30] have a neuroprotective potential in animal ischemic stroke models. In vitro or in vivo, our results showed that BMSCs preconditioned by NaHS could enhance secretion of BDNF and VEGF, uniformly. In fact, previous studies have shown that H₂S treatment induces VEGF and angiogenic factor after ischemic stroke and improves neurological outcomes [31]. In addition, H₂S upregulates the level of BDNF protein and blockage of BDNF-TrkB pathway reverses the protection of H₂S against cytotoxicity, oxidative stress, and apoptosis in neurons [32, 33]. Importantly, to determine whether

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**Figure 6: Administration of PcbMscs alleviated MACO-induced apoptosis, decreased Bax/Bcl-2 ratio and increased BDNF and VEGF expression.** (A) Representative photomicrographs (>20) of TUNEL staining of the brain tissues. Bar graphs showing quantification of TUNEL-positive cells. Scale bar = 50 μm. n = 4. (B) The protein and mRNA levels of Bax and Bcl-2 in injured cerebral tissues at day 3 and 14 after BMSCs transplantation were then analyzed by Western blot and semiquantitative RT-PCR, β-actin served as a loading control. Results were expressed as Bax/Bcl-2 ratio. n = 4. (C) The protein and mRNA levels of BDNF and VEGF in injured cerebral tissues at day 3 and 14 after BMSCs transplantation were then analyzed by Western blot and semiquantitative RT-PCR, β-actin served as a loading control. Each value was normalized to β-actin. n = 4. Values represent the mean ± SD *p < 0.05, **p < 0.01, ***p < 0.001 vs-PCBMSCs VS MACO; #p < 0.05, ##p < 0.01 PCBMSCs VS vs-PCBMSCs.
the bioactive factors released from H2S-preconditioned BMSCs provided protective effects on neurons subjected to injury, we treated MCAO animals with NaHS (1 μM) via tail vein in preliminary studies, the results showed that NaHS administration alone did not protect neurons from hypoxia-ischemia injury in vivo. Taking together these data and the results of the present study, we hypothesize that enhancement of paracrine effects of BMSCs for these neuroprotective factors may be indirectly resulted from improvement of the transplanted BMSCs survival due to preconditioning with H2S.

Angiogenesis can improve the micro-circulation in the zone of cerebral ischemia and inhibit the cell apoptosis. Moreover, the new blood vessels can induce the release of nutritional factors to promote the recovery of neurological function. In this study, we found that H2S-preconditioned BMSCs can promote the angiogenesis after cerebral infarction by CD31 staining, associated with enhanced secretion of VEGF. These results are similar to those in the previous studies [34, 35]. Thus it can be presumed that the reason for H2S-preconditioned BMSCs to recover the neurological function of animals with cerebral infarction may be the action to promote the angiogenesis. In addition, we found that hypoxia do not increase the VEGF expression in BMSCs, which is in contradiction with a numerous number of previous data [36, 37]. The discrepancy might be due to the severity of the insult and the time used in these studies.

In the cerebral ischemia, HIF-1α was increased and it induced the expression level of its downstream target genes, such as VEGF, erythropoietin, and CXC chemokine receptor 4 [38]. HIF-1α is also involved in angiogenesis, cell survival, anaerobic metabolism, cell migration, and differentiation, suggesting that HIF-1α plays an important role in the functional recovery of cerebral ischemia [38]. Previous studies showed that H2S increased HIF-1α and VEGF expression in vascular smooth muscle cells under hypoxic condition, which enhanced endothelial cell viability [39, 40]. Recently, one study showed that HIF-1α-induced VEGF overexpression in BMSCs protected neuron against brain ischemia [36]. Our findings are in agreement with these previous reports and revealed that BMSCs preconditioned by NaHS could enhance secretion of HIF-1α and VEGF, associated with a decreased apoptotic rate, suggesting that the NaHS-preconditioned BMSCs promote cellular survival through the regulation of VEGF by HIF-1α.

In this regard, it is important to note that a recent study indicated that therapeutic efficacy of rat BMSCs preconditioned with 200 μM NaHS added after myocardial ischemia in Sprague-Dawley rats [41], thus the used cells received much higher dose of H2S compared to the cells applied in our study. In our study NaHS at lower concentration can improve the survival of stem cells used for cell-therapy in the context of an in vitro cell therapy. Similar results were reported by other authors, who demonstrated that NaHS at 3 μM pretreatment can increase the survival of therapeutically used human adipose tissue-derived stem cells [42]. There are several important differences between these investigations. For example, in this study BMSCs were challenged to hypoxia for 72 h in 1% FBS medium, for the BMSCs failed to proliferate in serum-free conditions [43].

There are some important limitations to our study. First, whether H2S preconditioning change property of BMSCs which differentiate into neuronal lineage cells was not investigated. Second, how H2S up-regulates expression of BDNF and VEGF, and how these cytokines affect the neurological function were not determined. Finally, how the effects of H2S preconditioning using other routes/timing of stem cell delivery were not evaluated.

In conclusion, we have demonstrated that H2S preconditioning presented better neuron protection and an improvement of neurological function under hypoxic-ischemic conditions, which might provide a better therapy of BMSCs administration to cerebral ischemia. We have also elucidated the possible mechanisms of the beneficial effects of H2S preconditioned BMSCs acting, at least in part, through up-regulation of the survival of BMSCs and secretion of paracrine factors.

MATERIALS AND METHODS

Bone marrow mesenchymal stem cell cultures

Bone marrow mesenchymal stem cells (BMSCs) were harvested from 3 weeks Wistar rats as previously described [44]. Briefly, BMSCs were flushed out from the femoral and tibial bones of donor adult rats using a syringe and 20-gauge needle. The cells were suspended in DMEM/F12 supplemented with 10% FBS and incubated in 95% room air and 5% carbon dioxide at 37°C and nonadherent cells were removed by replacing the medium. The primary cultures were passaged at a ratio of 1:3 once the BMSCs reached 80–90% confluence. At passage 3, BMSCs were used for the present study.

Hypoxia-ischemic protocol

Prior to use in the experiment, plated cells were incubated with low serum (1% FBS) for 1 h. The medium was then replaced with low serum DMEM containing NaHS (Sigma-Aldrich) for the various time intervals and concentrations as indicated below. PI3K inhibitors (Wortmannin ) and ERK1/2-MAPK inhibitor (PD98059) were purchased from Sigma-Aldrich.

BMSCs were challenged to hypoxia by placing them in a chamber (Model: Heraeus HERAcell 240i; Thermo Scientific, USA) filled with 3% oxygen, 5% CO2, and 92% nitrogen at 37°C for the time intervals indicated below. BMSCs serving as controls were incubated at 37°C with 95% air and 5% CO2. Three or more different donor BMSCs were evaluated in triplicate for all experiments.
Cell viability assay

Cell viability was determined by the MTT assay. BMSCs were plated into 96-well culture plates at a density of 5 × 10^4 cells/ml with 200 μl culture medium per well. Following exposure to hypoxia with or without differing concentrations of NaHS, 20 μl MTT solution (5 mg/ml) was added to each well and incubated for 4 h. The medium was then aspirated and 200 μl dimethyl sulfoxide was added. The absorbance value was measured using a multiwell spectrophotometer (Bio-Rad, USA) at 490 nm. Cell viability was expressed as a percentage of viable cells obtained relative to that of controls.

Crystal violet assay

BMSCs were treated with test substances or vehicle for 72 h, and then fixed with 4% paraformaldehyde, stained with 0.5% crystal violet in 4% paraformaldehyde. The number of colonies was then counted. Colonies of less than 2 mm in diameter and faintly stained colonies were ignored [45].

Proliferation index by bromodeoxyuridine (BrdU) labeling

Cell proliferation was evaluated by BrdU incorporation assay. Dissociated BMSCs were transferred into 12-well plates with poly-D-lysine coated coverslips in medium. After this, BMSCs were treated with test substances or vehicle for 72 h and incubated with BrdU (10 μmol/l, Sigma-Aldrich) for 2 h. The cultured cells were fixed in 4% paraformaldehyde for 20 min, treated with 2 mol/l HCl at 37°C for 30 min, blocked with 10% normal goat serum for 30 min, and incubated with anti-BrdU monoclonal antibody (1:250; Sigma-Aldrich) overnight at 4°C. Goat anti-mouse IgG coupled to tetramethyl rhodamine iso-thiocyanate (TRITC) (1:200; Chemicon, Temecula, CA, USA) was used as the secondary antibody. The percentage of BrdU-positive cells over total DAPI cells was determined by randomly counting 10-nonoverlapping microscopy fields from each condition, in at least four independent experiments. The proportion of TUNEL-positive cells was expressed as the percentage of the total cells counted. Counting was performed in a blinded manner.

Detection of mitochondrial membrane potential (MMP or ΔΨm)

The loss of ΔΨm was determined using the JC-1 Mitochondrial Membrane Potential assay kit following the manufacturer’s instructions (Beyotime Institute of Biotechnology, China). Briefly, BMSCs were treated with test substances or vehicle for 72 h. Following treatment, BMSCs were washed with PBS and added JC-1 (5 μM), followed by incubation at 37°C for 20 min. Subsequently, BMSCs were washed twice with cold JC-1 staining buffer and visualized under a fluorescence microscope. The ratio of red to green fluorescence intensity was measured and data were expressed as relative to the mean sham value.

Ischemia model of middle cerebral artery occlusion

Adult male Wistar rats (270 to 300 g) were used for ischemic stroke experiments. A middle cerebral artery occlusion (MCAO) was induced by a modification of the intraluminal vascular occlusion method as previously described [47]. Briefly, Rats were initially anesthetized with 10% chloral hydrate. Rectal temperature was controlled at 37°C with a feedback-regulated water heating system. The right common carotid artery, external carotid artery (ECA), and internal carotid artery were exposed. A 3.0 monofilament nylon suture (18.5 mm, determined by animal weight), with its tip rounded by heating near a flame, was advanced from the ECA into the lumen of the internal carotid artery until it blocked the origin of the middle cerebral artery. At 2 h after occlusion, the animals were re-anesthetized and reperfused by withdrawing the suture until its tip cleared the lumen of the ECA.

Intravenous administration of BMSCs

NaHS was added to the cell culture medium (final concentration: 1 μM) for 72 h, followed by drug washout before transplantations. Then the deposits of single cell suspension in PBS (2 × 10^6 cells per ml) were pre-incubated with or without NaHS (final concentration: 1 μM) for 30 min before transplantation.

After 2 h MCAO and 24 h reperfusion, rats were randomly divided into three groups: MACO group (rats given MCAO without cell administration but with 1 ml PBS), non-PCBMSCs group (rats transplanted with 2 × 10^6 BMSCs), and PCBMSCs group (rats transplanted with 2 × 10^6 NaHS preconditioning-BMSCs) via tail vein. For intravenous injection, BMSCs (2 × 10^6) diluted in 1 ml PBS were injected slowly for five minutes in the tail vein. All transplantation procedures were performed under aseptic conditions.
Behavioral tests

All animals were assessed with modified neurological severity score (mNSS) tests [48] (Table 1) at day 1, 3 and 14 after BMSCs transplantation (n = 6 for each group). Neurological function including motor and sensory systems as well as reflexes and a balance test is graded on a numeric scale from 0 to 18 (normal score, 0; maximal deficit score, 18); the higher the score, the more severe the neurological deficit.

Cerebral infarction measurement

Cerebral infarction volume was measured by staining brain slices with triphenyltetrazolium chloride (TTC; Sigma) at day 14 after BMSCs transplantation. The animals (n = 3 for each group) were decapitated under deep anesthesia with 10% chloral hydrate (0.8 g/kg, i.p.). The brains were quickly removed and post-fixed in formalin. After dehydration with gradient ethanol, the brain tissues were embedded in paraffin and sliced into 3 sections/rat). The sections were dewaxed with a standard procedure and washed with PBS as described previously. The slides were counterstained with hematoxylin. The number of total and TUNEL-positive cells in the ischemic penumbra was calculated by counting six randomly selected microscopic fields in sections obtained from each rat (n = 4) at ×20 objective by a blinded observer. The percentage of TUNEL-positive cells against the total number of cells was calculated and averaged.

Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using the Trizol reagent (Gibco, Invitrogen) according to the manufacturer’s instructions. RNA concentration was determined using a spectrophotometer (Bio-Rad. Labs) at 260 nm. Identical amounts of RNA (2 μg) were reversely transcribed into cDNA using a commercial RT-PCR kit (Fermentas, Vilnius, Lithuania) according to the manufacturer’s instructions. cDNA was subsequently amplified by PCR with specific primers (Table 2). PCR products, separated on a 1.2% agarose/TAE gel, were visualized by staining with ethidium bromide. The densitometric calculations of these values were normalized to β-actin. The intensity of bands was determined using Image-Pro Plus 6.0 software.

Western blot analysis

Protein concentration in the supernatants of cells or ipsilateral cortex was determined using a BCA protein assay kit (Pierce Biotechnology, Inc.). A quantity of 30–50 μg of total proteins was loaded onto a 10–15% gradient polyacrylamide gel, electrophoretically transferred to a polyvinylidene difluoride membrane and probed with the following primary antibodies: Bax antibody (1:1000, Santa Cruz Biotechnology, CA, USA), Bcl-2 antibody (1:1000, Santa Cruz Biotechnology), Cleaved caspase-3 (1:500, Cell Signaling Tech. MA, USA), Caspase-3 (1:1000, Cell Signaling), Phospho-extracellular signal-regulated kinase (ERK)1/2
## Table 1: Modified neurological severity score (mNSS)

| Points | Motor tests                                                                 | Sensory tests                                                                 | Beam balance tests                                                                 |
|--------|------------------------------------------------------------------------------|------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|
| 3      | Raising rat by the tail                                                      | 1 Placing test (visual and tactile test)                                     | 0 Balances with steady posture                                                    |
| 1      | 1 Flexion of forelimb                                                        | 2 Proprioceptive test (deep sensation, pushing the paw against the table edge to stimulate limb muscles) | 1 Grasps side of beam                                                             |
| 1      | 1 Flexion of hindlimb                                                        |                                                                               | 2 Hugs the beam and one limb falls down from the beam                               |
| 3      | 1 Head moved > 10° to vertical axis within 30 s                               |                                                                               | 3 Hugs the beam and two limbs fall down from the beam, or spins on beam (> 60 s)  |
| 3      | Placing rat on the floor (normal = 0; maximum = 3)                           |                                                                               | 4 Attempts to balance on the beam but falls off (> 40 s)                            |
| 0      | Normal walk                                                                  |                                                                               | 5 Attempts to balance on the beam but falls off (> 20 s)                            |
| 1      | Inability to walk straight                                                   |                                                                               | 6 Falls off: No attempt to balance or hang on to the beam (< 20 s)                  |
| 2      | 2 Circling toward the paretic side                                           |                                                                               | Reflexes absent and abnormal movements                                             |
| 3      | 3 Fall down to the paretic side                                              |                                                                               | 1 Pinna reflex (head shake when touching the auditory meatus)                      |
| 2      |                                                                               |                                                                               | 1 Corneal reflex (eye blink when lightly touching the cornea with cotton)          |
| 1      |                                                                               |                                                                               | 1 Startle reflex (motor response to a brief noise from snapping a clipboard paper)|
| 4      |                                                                               |                                                                               | 1 Seizures, myoclonus, myodystony                                                 |
| 18     | Maximum points                                                               |                                                                               |                                                                                   |

One point is awarded for the inability to perform the tasks or for the lack of a tested reflex; 13 to 18 indicates severe injury; 7 to 12, moderate injury; 1 to 6, mild injury.

## Table 2: PCR primers used in this study

| Gene   | Forward (5′→3′) | Reverse (5′→3′) |
|--------|-----------------|-----------------|
| Bax    | GGT TGC CCT CTT CTA CTT TGC | TCT TCC AGA TGG TGA GCG AG |
| Bcl-2  | GGA TGA CTT CTC TCG TCG CTA C | TGA CAT CTC CCT GTT GAC GCT |
| BDNF   | AGC TGA GCG TGT GTG ACA GT | ACC CAT GGG ATT ACA CTT GG |
| VEGF   | GTG GAC ATC TTC CAG GAG TA | TCT GCA TTC ACA TCT GCT GT |
| β-actin| CTA TTG GCA ACG AGC GGT TCC | CAG CAC TGT GTT GGC ATA GAG G |
(1:2000, Cell Signaling), ERK1/2 (1:2000; Cell Signaling), Phospho-Akt (Ser 473) (1:500, Cell Signaling), Akt (1:1000; Cell Signaling), Brain-derived neurotrophic factor (BDNF, 1:1000, Santa Cruz Biotechnology), Vascular endothelial growth factor (VEGF, 1:100, Santa Cruz Biotechnology). β-actin (1:2000; Sigma-Aldrich) was used as an internal control. Secondary antibodies were horseradish peroxidase conjugated to goat/mouse anti-rabbit IgG (1:8000, Sigma-Aldrich). The membranes were developed using an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

Statistical analysis

Quantitative data were presented as the mean ± SD. Statistical analysis of data was performed with a one-way ANOVA using the *post-hoc* Tukey test for multiple comparisons of means. Differences were considered statistically significant if the *p* value was < 0.05.

Abbreviations

BDNF, brain-derived neurotrophic factor; BMSCs, Bone marrow mesenchymal stem cells; CNS, Central nervous system; DAPI, 4′, 6-Diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco’s modified Eagle medium; ELISA, Enzyme-Linked Immunosorbent Assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; H2S, hydrogen sulfide; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide sodium; MAPK, mitogen-activated protein kinase; NaHS, sodium hydrosulfide; PBS, phosphate buffered saline; RT-PCR, reverse transcription–polymerase chain reaction; VEGF, vascular endothelial growth factor.

ACKNOWLEDGMENTS AND FUNDING

This work was supported by funding from the Fundamental Research Funds of Shandong University (No.2015JC008); National Natural Science Foundation of China (No.81200879, 31300952); The Foundation for Outstanding Young Scientist in Shandong Province (No.2013BSE27020).

CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

Authors’ contributions

ZW was involved in study design, data interpretation and writing of the manuscript; DXL was involved in study design, data interpretation and manuscript editing; QZ performed the majority of the laboratory work and contributed to the analysis of data; SL, TL, HSL and XEW were responsible for the animal model; LY and FWW were responsible for cell culture; SLW was responsible for western blot. AJH was involved in manuscript editing. The authors have no conflict of interest to declare.

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