Carbon Monoxide Protects Neural Stem Cells Against Iron Overload by Modulating the Crosstalk Between Nrf2 and NF-κB Signaling

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Abstract
Although accumulating evidences have demonstrated pro-survival effects of CO against various insults, the precise mechanism explaining how neural stem cells (NSCs) are protected by CO also remains largely unknown. Here we report CO pro-survival effect on NSCs against iron overload was comparable to that obtained with pharmacological inhibitors of reactive oxygen species (ROS). Its pro-survival effect was accompanied by the inhibition of ROS and subsequent inhibition of NF-κB which is mediated through nuclear factor erythroid 2-related factor 2 (Nrf2), in that activation of Nrf2 by CO inhibited ROS via up-regulation of NQO-1 while down-regulation of Nrf2 reversed the pro-survival effect of CO both in vitro and in vivo. CO-mediated preconditioning results in Nrf2 up-regulation and NF-κB inhibition, suggesting that these two pathways act in an inverse manner to maintain redox homeostasis. Our findings revealed CO preconditioning as a promising treatment strategy to improve efficacy of NSCs transplantation after hemorrhagic stroke.

Keywords Apoptosis · Neural stem cell · Carbon monoxide · Stroke

Introduction
Neural stem cell (NSC) is considered to be a highly promising candidate for the therapy of hemorrhagic stroke (HS) because of its self-renew and multi-lineage differentiation characteristics. However, massive loss of donor cells post-engraftment becomes a major impediment that lessens the effectiveness of stem cells therapy. Donor cells in the deleterious microenvironment are subjected to various insults [1, 2]. In some studies, only 1–3% grafted cells survived after transplanting [3, 4]. Oxidative stress mediated damage in stem cells has gained more attention as one of the vital mechanisms implicated in the loss of implanted cells [5, 6]. Growing evidences indicate that there exists an oxidative component in HS-induced brain injury [7]. It has been well established that decomposition of red blood cells leads to iron overload which results in the accumulation of reactive oxygen species (ROS) after HS and leads to ruining the cellular redox-balance [8]. This event becomes a leading cause of brain damage and cell death after HS. Therefore, targeting iron overload-induced oxidative stress is a promising strategy to improve the clinical efficacy of NSCs therapy after HS.

CO has been recognized as an important gas-transmitter in the heart, lung, liver, and the central nervous system (CNS) [9]. In the CNS, CO regulates various signaling pathways in neurons, astrocytes, and stem cells, resulting in protective and anti-inflammatory effects after acute or chronic CNS injuries [10–12]. The development of CO-releasing molecules (CORMs) provides a safe and specific solution for CO delivering [13]. Previously, we showed CO decreased expression of cleaved caspase 3 via inhibiting NF-κB activation, which accounted for the protection against iron overload-induced damage in NSCs [14]. Nevertheless, the exact molecular mechanisms explaining how the NF-κB pathway is modulated by CO also remain to be established.

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Here, we aimed to elucidate whether and how the cyto-
protection by CO preconditioning is a result of the cross-talk
between Nrf2 and NF-κB signaling pathways that leads to
anti-oxidative and pro-survival effects.

Materials and Methods

CORM-2 (288144) and N-acetyl-cysteine (NAC) (A7250-10G) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CORM-2 was dissolved in dimethyl sulfoxide, and then diluted in culture media to achieve the required concent-
trations. Inactivated CORM-2 (iCORM-2) was prepared by dissolving CORM-2 under the same conditions for 3 days
at room temperature to liberate all CO from the molecule [14]. NSC C17.2, a stable, fully characterized, mouse stem cell line [15], was kindly provided by Prof. Jin WL (Instit-
ute of Nano Biomedicine and Engineering, Shanghai Jiao
Tong University, Shanghai, China). Ferrous chloride (FeCl2) was purchased from Sinopharm Chemical Reagent (Shang-
hai, China). Annexin V-FITC Apoptosis Detection Kit
(C1062), Diamidino-2-phenylindole dihydrochloride (DAPI)
(C10005) and Reactive Oxygen Species Assay Kit (S0033)
were from Beyotime Biotechnology (Shanghai, China). The
primary antibodies Nrf2 (C-20) (SC-722) and NF-κB P65
(SC-372) were from Santa Cruz Biotechnology (Santa Cruz,
CA, USA). Anti-NQO1 antibody (ab2346) was from Abcam
(Cambridge, UK). β-Actin (4970), Tublin (2148) and H3
(12167), were from Cell Signaling Technology (USA).

In Vitro Experiment

According to our previous study, we apply C17.2 cells, a cell
line originally cloned from mouse NSCs and immortalized
by v-myc, for all in vitro experiments. The concentration of
FeCl2 and CORM-2 were chosen according to our previous
study [14].

Cell Culture and Treatment

C17.2 cells were cultured in Dulbecco’s modified Eagle’s
medium (Invitrogen, USA) containing 10% (v/v) fetal bovine
serum (Gibco, Carlsbad, CA, USA), 5% (v/v) horse serum
(Gibco), and 2 mM L-glutamine (Gibco) at 37 °C in a humid-
ified incubator supplemented with 5% (v/v) CO2. C17.2 cells
were pretreated with or without 50 μM CORM-2/iCORM-2
for 6 h prior to stimulation with 500 μM FeCl2 for 24 h [14].

Western Blot Analysis

Western blotting was performed using standard techniques. Briefly, C17.2 cells were grown in 6-well cell culture plates
(2 × 105 cells/mL). After treatment, proteins were loaded on
8% (w/v) or 10% (w/v) SDS–polyacrylamide gel and protein
levels determined by Western blotting using Nrf2 (1:1000),
NF-κB p65 (1:1000), NQO-1 (1:1000) and HRP-conjugated
secondary antibody (1:5000). H3 (1:5000) antibodies, and
Tublin (1:5000) antibodies were used as marker proteins for
nuclear and cytosolic extracts respectively.

RNA Interference by Small Interfering RNA (siRNA)
of Nrf2

Pre-designed siRNAs against mouse Nrf2 and nontarget-
ing control-pool siRNA were purchased from GenePharma
(Shanghai, China). For Nrf2-siRNA transfection, C17.2
cells were grown in 6-well plates (2 × 105 cells per well)
until the confluence of cells reached approximately 50%.
The cells were then subjected to transient transfection with
N2h2-negative control siRNA, Nrf2-siRNA using the siRNA
transfection reagent Lipofectamine™ 2000, following the
manufacturer’s protocol. After 48 h, the transfected cells
were exposed to CORM-2/iCORM-2 for 6 h, followed by
lysis buffer for western blot analysis. Nontargeting siRNA
construct (NC) was used as negative control.

Intracellular Reactive Oxygen Species (ROS)
Measurement

Detection of intracellular oxidative states was performed by
using the fluorescent probe 2′,7′-dichlorofluorescein diac-
etate (DCFH-DA) [16]. Briefly, C17.2 cells were grown in
6-well cell culture plates (2 × 105 cells/mL) and pretreated
with CORM-2 (50 μM) or iCORM-2 for 6 h prior to stimu-
lation with 500 μM FeCl2 for 24 h. The DCFH-DA solu-
tion (10 μM) was added to the cells. After incubation with
DCFH-DA for 20 min at 37 °C, the fluorescence of DCF was
quantified using fluorescence microscopy at excitation and
emission wavelengths of 488 and 525 nm, respectively. ROS
production was expressed as a percentage of the control.
The fold-increase of ROS generation was compared with the
control cells, which were arbitrarily considered as onefold.

Flow Cytometry

Apoptosis was determined by using Annexin V-FITC apop-
tosis kits (Beyotime, China). The assays were performed
according to the manufacturer’s instructions. Briefly, the
cells were pretreated with CORM-2 (50 μM) or iCORM-2
for 6 h prior to stimulation with 500 μM FeCl2 for 24 h. After
pretreatment, single cell suspension was incubated with 5
μL of Annexin V-FITC and 10 μL of PI in 195 μL of binding
buffer for 15 min at room temperature in the dark. Then, the
rates of apoptosis were analyzed by a flow cytometry (BD
Biosciences, San Jose, CA). A minimum of 10,000 events
were acquired for each sample.
Immunofluorescent Staining

NSCs cultured on eight-well chamber slides poly-d-lysine-coated glass slips in 24-well dishes were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min. Then, cells were incubated for 1 h in blocking solution (PBS containing 3% bovine serum albumin and 0.3% Triton X-100). Cells were then incubated with antibodies against Nrf2 (1:200), NF-κB p65 (1:200) at 4 °C overnight. After three washes, samples were then washed with PBS and cover slipped with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlington, ON, Canada) nuclear counterstain. Samples were mounted onto slides with anti-fade solution and examined under a confocal laser-scanning microscope.

In Vivo Experiments

We used primary cultures of RFP Tg-NSCs for transplanting in vivo experiments. The diagram for the in vivo experiment was shown in Fig. 7A.

Animals

All animal procedures were carried out in accordance with National Institutes of Health guide for the care and use of Laboratory animals. Donate NSCs were harvested from homozygous red fluorescent protein transgenic (RFP Tg) mice (C57BL/6-RFP Tg and Nrf2 knockout (Nrf2−/−) C57BL/6-RFP Tg mice (Shanghai Jiaotong University Med-X center, Nanjing, China). Wild-type C57BL/6 mice (Slac laboratory animal CO.LTD, Shanghai, China) were used for HS models. All animals were housed on a 12:12-h light/dark cycle with environmental temperatures at 18–22 °C. Food and water were freely available.

Isolation and Culture of Fetal Neural Stem Cells

NSCs were isolated from the subventricular zones of RFP Tg fetal mice on the 14th day of gestation, as described previously [17]. In brief, bilateral subventricular zones were dissected and mechanically dissociated. After collection, the cells were resuspended in Neuro-basal-A medium (Invitrogen, Carlsbad, CA, USA) containing B-27 supplement (Invitrogen), l-glutamine (Invitrogen), 20 ng/mL mouse fibroblast growth factor-basic (PeproTech, Rocky Hill, NJ, USA), and 10 ng/mL mouse epidermal growth factor (PeproTech, Rocky Hill, NJ, USA). Cells were grown as suspending neurosphere. The medium was half-changed every 2 days and cells were passaged weekly. Cells that had been passaged 5 to 10 times were used for the experiments.

HS Model and Experiment Group

We used an experimental HS procedure, described previously [2]. Briefly, male C57BL/6 mice (8 weeks old, 20 to 25 g) were anesthetized with ketamine/xylazine (100 mg/10 mg/kg, Sigma, St. Louis, MO), and positioned in the stereotactic frame. After a midline scalp incision, a hole was drilled in the right side of the skull (0.0 mm anterior and 2.5 mm lateral to the bregma). Blood (20 μL) without any anticoagulant was collected from the tail tip for injection. A 30-gauge needle attached to a 50-mL Hamilton micro-syringe was inserted 3.5 mm ventral from the surface of the skull. 10 μL of blood was injected over 10 min. The remaining 10 μL of blood was injected using the same procedure after 5 min. After injection, the needle was left in place for 25 min and then slowly removed.

Experimental groups were non-precondition group, transplantation of RFP-NSCs (3*10^5/2 μL; n = 15); iCORM-2-precondition group, transplantation of iCORM-2 preconditioned RFP-NSCs (3*10^5/2 μL; n = 15) and CORM-2-precondition group, transplantation of CORM-2 preconditioned RFP-NSCs (3*10^5/2 μL; n = 15); CORM-2-precondition Nrf2−/− NSCs group, transplantation of CORM-2 preconditioned Nrf2−/− RFP-NSCs (3*10^5/2 μL; n = 15). All methods and assessments described below were carried out by individuals blinded to the groups.

CO Preconditioning and Intracerebral Transplantation

CORM-2/iCORM-2 dissolved in DMSO was added to the cell culture medium (final concentration: 50 μM) for 6 h prior to transplantation, followed by drug washout before transplanting. NSCs were transplanted on the third day after HS as described previously [2].

Assessment of Survival of Donate NSCs

The animals were sacrificed on the 30th days after transplantation. The survival of donate RFP Tg NSCs were assessed on 12 serial coronal sections per brain (0.25 mm apart) using unbiased computational stereology.

Statistical Analysis

Data was analyzed by using SPSS version 18 software. The significance of the difference among mean values was determined by the Student–Newman–Keuls test or two-way
analysis of variance followed by the Bonferroni post hoc test. p values < 0.05 were accepted to be statistically significant.

Results

The ROS Role in Apoptosis of NSCs (C17.2 Cells) Challenged by Iron Overload

The present investigation was aimed to determine the role of ROS in iron overload-induced apoptosis in NSCs. After 24 h of iron overload, flow cytometry analysis revealed a significant increase of apoptosis of NSCs (C17.2 cells) up to 21.4 ± 3.0% vs control cells 1.9 ± 0.4% (p < 0.05) (Fig. 1A, B, D). As the results showed in Fig. 1, co-treatment with ROS inhibitor N-acetylcysteine (NAC 1 mM) protected NSCs against iron overload-induced cell apoptosis and the apoptosis rate decreased from 21.4 ± 3.0% to 7.5 ± 0.8% (p < 0.05) (Fig. 1B, C, D).

Iron Overload Induced Activation of NF-κB P65 Signaling Pathway via ROS in NSCs (C17.2 Cells)

Our previous study demonstrated iron overload induced significant activation of NFκB P65 [14]. To confirm the role of ROS on iron overload-induced NFκB activation, immunofluorescence was performed to detect the nuclear translocation of NFκB P65 (Fig. 2A). We found that NFκB P65 was tethered in cytoplasm in un-stimulated control C17.2 cells, but in those cells challenged by FeCl₂, the nuclear translocation of NFκB P65 was significant. In NSCs (C17.2 cells) co-treated with ROS inhibitor (NAC 1 mM) the translocation was inhibited. For further confirmation of ROS role, next we performed Western blot and examined the changes in NFκB P65. We found that iron overload-induced up-regulation of...
NFκB P65 in nucleus fraction was substantially suppressed by NAC (Fig. 2B, C). These findings strongly demonstrated that ROS signaling cascades are involved in the regulation of iron overload-induced NFκB P65 activation.

CO Inhibits Iron Overload-Induced Intracellular ROS Accumulation in NSCs (C17.2 Cells)

ROS are known to play a key role in the damage of neural cells after HS. Our studies demonstrated that iron overload-induced ROS accumulation in NSCs may mediate the increase of apoptosis of NSCs. As depicted in Fig. 3, NSCs (C17.2 cells) subjected to FeCl₂ (500 μM) for 24 h triggered the intracellular ROS production (p < 0.001) (Fig. 3A, B, E). However, the aberrant accumulation of ROS was reduced by preconditioning with CORM-2 (50 μM) prior to iron overload stimulation (p < 0.05) (Fig. 3D, E). This finding suggests that resistance to oxidative stress is main mechanism of CO-mediated protection.

CO Triggers the Nuclear Translocation of Nrf2 and Up-Regulates the Level of Nrf2 in Nucleus in NSCs (C17.2 cells)

Nrf2 is an important transcription factor, which mediate antioxidant responses upon stimulation. Here we examined whether CO suppressed the iron overload-induced ROS accumulation via activation of Nrf2. Nrf2 is normally located in the cytosol, bound and inhibited by Keap1. Following activation, Nrf2 is released from this inhibition, and translocates to the nucleus and activates transcription of downstream antioxidant genes. To verify this, immunofluorescence was performed to detect the nuclear translocation of Nrf2. We found that Nrf2 was tethered in cytoplasm in control cells, but in those cells preconditioned with CORM-2 such translocation is significant (Fig. 4A).
For further confirmation of CO anti-oxidant effects, next we performed Western blot and examined the changes in nuclear and cytosolic fractions of NSCs. Interestingly, Consistent with nuclear translocation of Nrf2 induced by CO, a significantly increase of the level of Nrf2 in nucleus fractions about 2–3-fold was determined by western blot (Fig. 4B, C).

**CO Protective Effects are Associated with Enhanced Antioxidant Genes in NSCs (C17.2 Cells)**

We next investigated whether CO could stimulate Nrf2 mechanism, with its transcriptional activity being related to phase II antioxidant enzyme genes such as NQO-1 which are responsible for scavenging ROS [18]. We hypothesized that protective effect of CO may be due to induction of antioxidant genes, NQO-1 through the typical Nrf2/ARE-signaling cascades. To confirm this, first we measured the time dependent changes in proteins levels of NQO-1after CO preconditioning. As expected, CO preconditioning significantly increased NQO-1 protein expressions in a time dependent manner (Fig. 5A). The significantly increased NQO-1 protein after CO preconditioning started after treatment for 6 h (Fig. 5A). CO preconditioning increased NQO-1 significantly in NSC challenged by FeCl₂ (Fig. 5B). Next we down-regulate Nrf2 to confirm whether CO could promote antioxidant genes via Nrf2/ARE signaling pathway. Results showed Nrf2-siRNA significantly down-regulated CO-mediated protein expression of Nrf2, NQO1 (Fig. 5C).

**CO Suppresses NFκB Activation Through Up-Regulation of Nrf2 in NSCs (C17.2 Cells)**

It has been reported that CO pretreatment can suppress the activation of NFκB. To delineate the role of Nrf2 signaling cascade on the fate of NFκB, we examined nucleus fraction protein level of NFκB in NSCs transfected by siRNA Nrf2. We found that NFκB increased significantly and the inhibition of NFκB by CO was abolished in nucleus fraction after Nrf2 was knocked down (Fig. 5D). These findings illustrate that CO-mediated suppression of iron overload-induced NFκB activation was regulated by activation of Nrf2.

**Nrf2 Knockdown Diminishes the Protective Effects of CO in Iron Overload Stimulated NSCs (C17.2 Cells)**

To reveal the importance of Nrf2 activation behind CO-induced protection against iron overload, we developed an Nrf2 knockdown C17.2 cells using siRNA transfection. The apoptosis of NSCs was determined by flow cytometry. CO decreased the apoptosis of NSCs induced by iron overload from 24.4 ± 4.4% to 7.5 ± 1.0% (Fig. 6A, B, D, F). However, CO-mediated protection was abolished in the cells transfected by siRNA Nrf2 with an apoptosis even up to 38.6 ± 3.1% (Fig. 6E, F). The protective effect was exclusively for CO because pretreatment with iCORM-2 did not cause any additional effect, i.e. the apoptosis of NSCs were unmodified (Fig. 6B, C, F). Concomitant with the apoptosis of NSCs, CO-induced up-regulation of Nrf2 and NQO-1 proteins was barely
Fig. 4 Effects of CO preconditioning on Nrf2 signaling pathway in NSC challenged by iron overload in vitro. A Representative images showing CO preconditioning (50 μM CORM-2) triggered activation of Nrf2. Double staining with DAPI (blue) and Nrf2 (red). B, C Representative bands and quantification of Nrf2 in the cytoplasm and nuclear fraction (*p < 0.05 vs. the FeCl2 group or iCORM-2 group). Data represent the mean ± SD. Bar 25 μm
observed in Nrf2 knockdown cells (Fig. 5C). These data suggest that induction of NQO-1 by CO requires Nrf2 activation, which seems to be essential behind CO antioxidant effects against iron overload.

**Quantification of Survival of the Transplanted Red Fluorescent Protein-Positive Neural Stem Cells In Vivo**

Altogether, 60 HS mouse survived with 15 in each group. The transplanted RFP-positive cells were counted on 12 serial coronal sections per brain as described [2]. Consent to the previous studies, non-preconditioning NSCs demonstrated a significantly low survival on the 30th day after transplantation (Fig. 7B, F). CO preconditioning NSCs showed a significant improvement of survival in HS mouse (Fig. 7D, F) while pretreatment with iCORM-2 did not cause any additional effect (Fig. 7C). The pro-survival effect of CO was reversed by knocking out Nrf2 (Fig. 7E).

**Discussion**

For the first time we provide in vitro and in vivo evidences to demonstrate that CO protects implanted NSCs against iron overload-induced redox imbalance in HS. Our results show that CO effectively enhances the survival of NSCs after HS which is associated with reestablishing the redox balance via modulation of the crosstalk between Nrf2 and NF-κB signaling pathway.

It has been well established that iron overload was responsible for the adverse outcome in stem cell [19, 20]. In our study, when NSCs were treated with FeCl₂ for 24 h, we found a marked cellular toxic effect consistent with a sustained increase of ROS. Under these experimental conditions, nuclear protein level of NF-κB P65 was increased concomitant with significant translocation. The activation of the NF-κB signaling pathway seems to be responsive to excessive ROS generation, thus affecting the redox sensitive NF-κB signaling pathway and activation of NF-κB P65;
produce more ROS which seems to regenerate positive feedback [21]. Indeed, considerable evidences established the link between oxidative stress and NF-κB which was mainly from the inhibition of NF-κB activation by cellular antioxidants [22, 23]. Further evidence in our study showed that iron overload-induced NF-κB P65 activation was inhibited by N-acetylcysteine (NAC) (pharmacological inhibitors of ROS), which implies that iron overload-mediated NF-κB activation in NSC was ROS dependent. A growing body of evidences have demonstrated the deleterious role of the activation of NF-κB induced by iron overload [24, 25]. Such deleterious role of the activation of NF-κB has also been shown in PC12 cell and hippocampal neuron cell challenged by other oxidant regent [26]. The neuroprotective effect of inhibition of NF-κB was found in NSCs in our research and reproduced in other study [27].

It has been well established that CO has a role in preconditioning as low doses can protect against inflammation and apoptosis. Our previous data also indicated pretreatment with CO could inhibit the activation of NF-κB P65 induced by iron overload. Such inhibition was reasoned by the suppression of ROS as evidenced by suppressed ROS in NSCs upon the preconditioning with CO and suppressed NF-κB activation upon treatment via inhibition of ROS with NAC. In this regard, CO protective effects are similar to that of aspirin, which has been shown to attenuate H2O2-induced apoptosis in Hela cell through NF-κB inactivation [28]. The present findings further emphasized that CO-mediated suppression of NF-κB activation is associated with the inhibition of iron overload-induced ROS accumulation in NSCs. It is interesting that CO can also activate heme-oxygenase1 (HO-1) which in turn can catalyze heme to generate ferrous iron. HO-1has been recognized as an important protective mechanism against various insults, however, the effect of HO-1in HS is still controversial [29]. Growing evidences demonstrate the effect of HO-1in intracranial hemorrhage tends to be harmful [30]. We believe the protective effect of CO preconditioning in HS is an overall effect.

The Nrf2 signaling pathway is the main pathway responsible for cell defense against oxidative damage and...
maintaining the cellular redox balance [31]. Disruption of Nrf2-mediated Nrf2/ARE-signaling pathway could exacerbate the oxidative damage [32]. There are growing evidences that CO acts in CNS by activation of Nrf2 signaling [33, 34]. In our study, the activation of Nrf2 was evidenced by the significant up-regulation of the nuclear protein level of Nrf2 and the nuclear translocation of Nrf2. Elevated antioxidant enzymes levels by CO through Nrf2/ARE cascades may play a frontline of defense against iron overload-induced ROS. The expressing of NQO-1 in NSC exhibited in a time-dependent manner after CO preconditioning. The pivot role of NQO-1 induced by Nrf2 in NSC against oxidative stress has been well established [18]. Nrf2 knockdown studies confirmed this phenomenon by detecting the down-regulation of NQO-1 and abolished the pro-survival effect of CO both in vitro and in vivo.

Recently, substantial efforts have been put on the issue that when Nrf2 talks who is listening. It is worthwhile to note that suppression of NF-κB accumulation by CO was accompanied by activation of Nrf2. Evidences have also been accumulating that suggest that activation of Nrf2 antioxidant signaling occurs concomitantly with suppression of NF-κB response. [35, 36]. Furthermore, Nrf2−/− mice has been demonstrated increased NF-κB activation when compared with wild-type after traumatic brain injury [37]. These findings further emphasize the crosstalk between NFκB and Nrf2 transcription factors [38]. The relationship between Nrf2 and NF-κB signaling pathway is now suggested as an important cross-talk mechanism by which Nrf2 can overcome apoptosis and provide protection against various deleterious stresses [39, 40]. Several lines of evidence suggest that Nrf2 regulates NF-κB activation through maintenance of redox homeostasis. In the presence of CO, Nrf2 protein expression was up-regulated concomitant with increase of nuclear translocation, which leads to the induction of antioxidant enzymes NQO1 with simultaneous inhibition of iron overload-induced ROS production and a subsequent suppression of the NF-κB. Nrf2-mediated expression of NQO1 has been demonstrated efficiently to neutralize ROS and to cause

![Fluorescent staining with RFP (red)](image)

**Fig. 7** NSCs survival in vivo. Fluorescent staining with RFP (red) revealed that the grafted cells surrounded the HS lesion on the 30th day after transplantation. A. Diagram for the in vivo experiment. B, F Low NSCs survival in non-preconditioned group; C, F Low NSCs survival in iCORM-2-preconditioned group; D, F significantly more NSCs survival in CO preconditioned group (*p < 0.05 vs. non-preconditioned group or iCORM-2-preconditioned group or CO preconditioned Nrf2−/− NSCs group); E, F low NSCs survival in CO preconditioned Nrf2−/− NSCs group.
the reduction of ROS-mediated NF-κB activation [41]. Notably, knocking down Nrf2 in NSCs also witnessed that iron overload-induced elevation of NF-κB levels remains higher and more significant cell apoptosis occurs. Since Nrf2-regulated genes are responsible for increased cellular oxidative stress and ROS is involved in iron overload-induced activation of NF-κB, we postulate that Nrf2 activation by CO may, at least in part, down-regulate the iron overload-induced NF-κB through the modulation of redox status. In accordance with our findings, Minellia et al. have shown that regulation of the crosstalk between Nrf2 and NF-κB by CO is highly neuroprotective by reducing oxidative stress in PC12 and murine microglial BV2 cells [42]. These findings underlined the possibility that CO-mediated neuro-protective effect may be achieved by the modulation of the crosstalk between Nrf2 and NF-κB via reestablishing the redox balance in NSC.

On particular interest of our in vitro findings, we conducted in vivo studies to demonstrate the therapeutic potentials of CO pretreatment in HS mouse model. The pro-survival effect of CO was further endorsed by in vivo evidences. In our experiment, survival of more CO preconditioned RFP-NSCs ascertains the protective role of CO, however, knocking down Nrf2 of NSCs reverses the protection of CO. Also, day 3 after HS is an important time point for hematoma evacuation operation in clinic, and NSCs could be implanted at the same time of hematoma evacuation. SO, day 3 after HS is an interesting time point for investigation of the survival of donor NSCs and it has potential implication for clinical therapy [43].

Conclusion

Our findings demonstrated that CO preconditioning programs NSCs to tolerate oxidative stress induced by iron overload after HS. The anti-oxidant effects of CO are mediated through the activation of Nrf2 and subsequent up-regulation of antioxidant genes NQO-1. Our data directly implies that CO protects NSCs against iron overload by modulating the crosstalk between Nrf2 and NF-κB via reestablishing intracellular redox balance. Through this better understanding of CO-mediated signal transduction and gene regulations involved in redox status, our findings may provide an opportunity to improve the efficacy of NSCs therapy after HS.

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Data Availability All associated data are available on reasonable request.

Declarations

Conflict of interest All authors declare no conflict of interest.

Ethical Approval The study was approved by the Ethical committee of The Affiliated Wuxi No. 2 Peoples’ Hospital of Nanjing Medical University, Wuxi, China.

Consent to Participate and Consent for Publication All authors consent to participate and consent for publication.

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