Sirt1 Mediates Improvement in Cognitive Defects Induced by Focal Cerebral Ischemia Following Hyperbaric Oxygen Preconditioning in Rats

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Summary

Hyperbaric oxygen preconditioning (HBO-PC) has been proposed as a safe and practical approach for neuroprotection in ischemic stroke. However, it is not known whether HPO-PC can improve cognitive deficits induced by cerebral ischemia, and the mechanistic basis for any beneficial effects remains unclear. We addressed this in the present study using rats subjected to middle cerebral artery occlusion (MCAO) as an ischemic stroke model following HBO-PC. Cognitive function and expression of phosphorylated neurofilament heavy polypeptide (pNF-H) and doublecortin (DCX) in the hippocampus were evaluated 14 days after reperfusion and after short interfering RNA-mediated knockdown of *sirtuin1* (*Sirt1*). HBO-PC increased pNF-H and DCX expression and mitigated cognitive deficits in MCAO rats. However, these effects were abolished by *Sirt1* knockdown. Our results suggest that HBO-PC can protect the brain from injury caused by ischemia-reperfusion and that Sirt1 is a potential molecular target for therapeutic approaches designed to minimize cognitive deficits caused by cerebral ischemia.

Key words

HBO-PC • Sirt1 • MCAO • Cognitive defects

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Introduction

Stroke is a leading cause of morbidity and mortality worldwide (Russo *et al.* 2011) that can lead to severe neurological disabilities in adults. In addition to motor and sensory disturbances (Meyer *et al.* 2015), stroke is associated with cognitive deficits involving spatial learning and memory acquisition and retention (Massa *et al.* 2015, Jafari *et al.* 2016, Kraft *et al.* 2015). Further investigation of the underlying pathogenesis mechanism of stroke and development novel treatment strategies might be benefit for the therapy of stroke.

During ischemia and reperfusion, elevated levels of reactive oxygen species activate diverse signaling pathways, resulting in oxidative stress (Niizuma *et al.* 2009, Deb *et al.* 2010) and inflammation (Minami *et al.* 2006); systemic administration of antioxidants or anti-inflammatory agents can improve ischemic neurological damage, decrease infarct size, prevent hemorrhagic transformation, and improve neurological and cognitive functions (Li *et al.* 2011a, Du *et al.* 2012, Qiao *et al.* 2012, Kure *et al.* 2016). Thus, oxidative stress and inflammation are major contributors to post-ischemic injury (Chan 1996, Amantea *et al.* 2009, Jin *et al.* 2010, Chen *et al.* 2011).

Hyperbaric oxygen preconditioning (HBO-PC) has been proposed as a safe and practical approach for neuroprotection against ischemic stroke (Xiong *et al.* 2000). HBO-PC was shown to decrease infarct size, prevent hemorrhagic transformation, and improve neurological function in hyperglycemic middle cerebral artery occlusion (MCAO) rats (Soejima *et al.* 2013, Bian *et al.* 2015). Previous studies showed that sirtuin1 (Sirt1) which is involved in memory formation, brain plasticity, axonal protection, and neuronal survival (Kim *et al.* 2007, Gao *et al.* 2010) mediates HBO-PC-associated ischemic tolerance in rat brain (Yan *et al.* 2013). However, the outcomes of HBO-PC on the cognition deficits as well as the precise role of Sirt1 in neuroprotection are not well understood.

We addressed this in the present study using a rat model of ischemic stroke in which the animals were subjected to HBO-PC prior to MCAO; then the cognitive function, changes in hippocampal structure, and the expression of phosphorylated neurofilament heavy polypeptide (pNF-H) and doublecortin (DCX) were evaluated 14 days later. We also examined the role of Sirt1 by short interfering (si) RNA-mediated knockdown of *Sirt1* transcript.

Materials and Methods

Animals

Animal experiments were approved by the Ethics Committee for Animal Experimentation and were carried out in accordance with the National Institutes of Health (NIH) Guidelines for Care and Use of the Laboratory Animals (Bethesda, MD, USA). Male Sprague Dawley (SD) rats weighing 280-320 g were obtained from the animal center of Fourth Military Medical University. Four rats were housed per cage in an air-conditioned room (at 22 ± 1 °C) with 50-55 % relative humidity under a 12-h light/dark cycle and provided with food and water *ad libitum*.
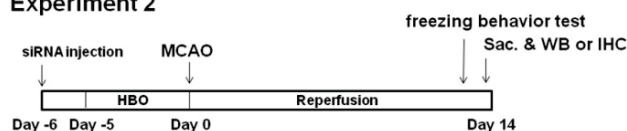
Experiment 1

After 7 days of acclimatization, rats were randomly assigned to one of the following four groups (n=12 each): sham, HBO, MCAO, and HBO + MCAO. Freezing behavior was evaluated 14 days after MCAO; the animals were then sacrificed, and hippocampal expression of pNF-H and DCX was evaluated by immunohistochemistry and Western blotting.
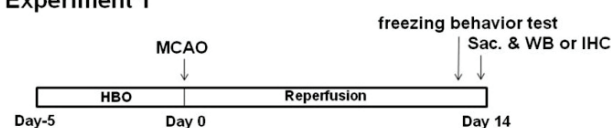
Fig. 1. Schematic diagram detailing the time course of treatment.

Experiment 2

After acclimatization, rats were randomly assigned to four groups (n=12 each): siRNA-C, HBO + siRNA-C, siRNA-Sirt1, and HBO + siRNA-Sirt1. Rats in the siRNA-C and HBO + siRNA-C groups were transfected with control siRNA, while those in the siRNA-Sirt1 and HBO + siRNA-Sirt1 groups were transfected with Sirt1-siRNA; the animals then underwent sham HBO or HBO treatment 24 h later and were subjected to MCAO 24 h after the last HBO treatment. Freezing behavior and hippocampal expression of pNF-H and DCX were evaluated 14 days after MCAO (Table 1).

Table 1. The description of the abbreviation of each group.

Group abbreviation	Treatments
siRNA-C	Transfected with control siRNA then underwent sham HBO treatment and subjected to MCAO
HBO + siRNA-C	Transfected with control siRNA then underwent HBO treatment and subjected to MCAO
siRNA-Sirt1	Transfected with Sirt1-siRNA then underwent sham HBO treatment and subjected to MCAO
HBO + siRNA-Sirt1	Transfected with Sirt1-siRNA then underwent HBO treatment and subjected to MCAO

HBO-PC

The protocol for HBO-PC is described in our previous studies (Yan *et al.* 2011, Yan *et al.* 2013). Briefly, rats were exposed HBO at 2.5 ATA in 100 % oxygen for 1 h daily for 5 consecutive days in a hyperbaric chamber (model no. NG90-IIC; Yantai Binglun, Yantai, China) maintained between 23 °C and 26 °C. Compression and decompression were carried out at a rate of 0.2 atm/min. The total time that animals spent within the chamber was approximately 1 h + compression (12.5 min) + decompression (12.5 min) and nearly 20 rats were exposed to HBO during one session and oxygen concentration was monitored with a calibrated oximeter. Accumulated CO_2 was absorbed by calcium carbonate crystals. The exposure was initiated at the same time each day in order to minimize the impact of biological rhythms. Rats under normobaric conditions in the same chamber served as controls.

Induction of transient focal cerebral ischemia

Focal cerebral ischemia was induced as described in previous studies using the method of right MCAO with an intraluminal filament (Wang *et al.* 2009). Briefly, rats were anesthetized by intraperitoneal injection of 40 mg/kg

sodium pentobarbital and placed on a heating pad that maintained their body temperature. After making a midline neck incision, the right common and external carotid arteries were exposed and proximally ligated. A 3-0 rounded-tip nylon monofilament suture (1893G; Ethicon, Somerville, NJ, USA) was advanced from the common carotid artery incision just below the carotid bifurcation up to the internal carotid artery until regional cerebral blood flow was reduced to <16 % of the baseline level, thereby occluding the base of the anterior and middle cerebral and posterior communicating arteries. After 2 h, the filament was slowly withdrawn and blood flow was restored to ≥75 % of baseline. Cerebral blood flow was monitored by laser Doppler flowmetry (PeriFlux System 5000; Perimed, Järfälla, Sweden) in the ipsilateral cortex (2 mm posterior and 5 mm lateral to bregma) and rectal temperature was monitored (90303B; Spacelabs Healthcare, Snoqualmie, WA, USA) and maintained between 37.0 °C and 37.5 °C. Sham rats underwent the same surgical procedure but without MCAO. There were no significant differences between cerebral blood flow values before, during and after MCAO between MCAO and HBO + MCAO group, and among siRNA-C, HBO + siRNA-C, siRNA-Sirt1 and HBO + siRNA-Sirt1 as well (Table 2).

Table 2. The cerebral blood flow values (PU) of each group.

Group	Before MCAO	During MCAO	After MCAO
MCAO	140.00 ± 8.59	20.30 ± 1.24	109.20 ± 6.70
HBO + MCAO	142.25 ± 10.74	20.63 ± 1.56	110.96 ± 8.38
siRNA-C	141.38 ± 8.35	20.50 ± 1.21	110.27 ± 6.51
HBO + siRNA-C	143.50 ± 11.60	20.81 ± 1.68	111.93 ± 9.05
siRNA-Sirt1	152.25 ± 12.33	22.08 ± 1.79	118.76 ± 9.61
HBO + siRNA-Sirt1	144.13 ± 12.05	20.90 ± 1.75	112.42 ± 9.40

SiRNA transfection in rat brain

In vivo siRNA transfection in rat brain was carried out as previously described (Chen *et al.* 2009, Xue *et al.* 2016). Briefly, rats were anesthetized with 10 % chloral hydrate, and a stainless steel cannula was stereotaxically implanted into the cerebral ventricle. The stereotaxic coordinates were 1.8 mm posterior and 1.5 mm lateral to bregma at a depth of 4.0 mm from the skull surface. A 10-µl volume of 50 nmol/l Sirt1 siRNA (sc-108043) or control siRNA (sc-37007) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) was delivered into the lateral ventricle.

Assessment of freezing behavior

The experiment was performed as previously described (Nie *et al.* 2014) in the shock chamber and neutral test contexts. For shock application, rats were placed in the shock chamber, and after 196 s, a single 4-s scrambled electric shock was delivered in the form of a 1-mA current to the feet through a metal grid, which produced signs of pain (jumping or vocalization). Stressed rats remained in the shock chamber for another 60 s before being returned to their home cage. To assess conditioned fear, rats were re-exposed to the shock chamber for 3 min without tone presentation or further

shock application and then immediately returned to their home cage. To test for fear sensitization, animals were placed in the neutral test chamber and after 3 min a neutral tone (80 dB, 9 kHz) was presented for 3 min. After another 60 s, rats were returned to their home cages. The activity of each rat was recorded and analyzed using the Freezing Scan system (Clever Sys, Reston, VA, USA).

Western blotting

The hippocampus was dissected and lysed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer composed of 62.5 mM Tris-HCl, 2 % w/v SDS, 10 % glycerol, 50 mM dithiothreitol, and 0.1 % w/v Bromophenol Blue. After homogenization, tissue extracts were centrifuged at $12,000 \times g$ for 10 min. The protein concentration in the supernatant was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Aliquots of lysate with loading buffer (Beyotime Institute of Biotechnology, Beijing, China) were boiled for 10 min and then cooled to room temperature, and equal amounts of protein (20-40 mg) were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) that was blocked with 5 % skim milk in Tris-buffered saline and then incubated overnight at 4 °C with the following primary antibodies: rabbit anti-DCX (1:500, ab18723; Abcam, Cambridge, UK), mouse anti-pNF-H (1:800, ab7795; Abcam), and mouse anti-β-actin (1:10,000, A5441; Sigma-Aldrich, St. Louis, MO, USA). After washing, the membrane was incubated with the appropriate horseradish peroxidase-conjugated IgG for 1 h at room temperature. Immunoreactivity was visualized on X-ray film using Super ECL Plus detection reagent (34077; Thermo Fisher Scientific, Waltham, MA, USA). Band intensity was normalized to β-actin level using ImageJ software (NIH).

Immunohistochemistry

Immediately after behavioral testing, half of the rats from each group were deeply anesthetized with chloral hydrate and perfused through the ascending aorta with 0.1 M phosphate buffered saline (PBS) followed by 4 % paraformaldehyde in PBS. The brains were removed and post-fixed in the same solution for 1 h, then dehydrated in 25 % sucrose in PBS at 4 °C until saturation. Serial coronal sections were cut at a thickness of 16 μm on a sliding microtome (CM3050 S; Leica, Buffalo Grove, IL, USA) and mounted on gelatinized slides. Three sets of sections from each rat brain were used for immunofluorescence labeling. Sections were incubated for 24 h at room temperature with a primary antibody against pNF-H (1:500) or DCX (1:300) in blocking solution composed of 0.2 % Triton X-100 and 5 % bovine serum albumin (Sigma-Aldrich) in PBS. Sections were then incubated with Alexa Fluor 488 donkey anti-rat (1:1,000, ab150073; Abcam) or anti-mouse (1:1,000, ab150105; Abcam) IgG (Invitrogen, Carlsbad, CA, USA) at room temperature for 3 h. Every sixth section throughout the hippocampus was processed and visualized under a laser scanning confocal microscope (FV-1000, Olympus), and the integral optical density (IOD) of pNF-H or DCX immunofluorescence in the images was analyzed with Image-Pro plus software (Media Cybernetics, Rockville, MD, USA).

Statistical analysis

Data were analyzed with SPSS v.16.0 software (SPSS Inc., Chicago, IL, USA) and are presented as mean ± SEM. Differences between groups were evaluated by one-way analysis of variance and a *post hoc* least significant difference (LSD) test. Differences were considered significant at $P<0.05$.

Results

*HBO-PC reduces cognitive deficits induced by cerebral ischemia*

Significant differences were observed between groups for both conditioned fear ($F_{3,21}=9.639$, $P<0.01$) and sensitized fear ($F_{3,26}=13.08$, $P<0.01$) (Fig. 2). Multiple comparisons revealed that MCAO caused
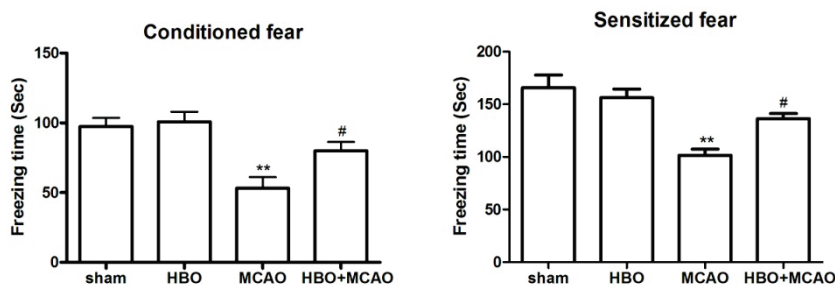
Fig. 2. HBO-PC improves cognitive behavior in the conditioned fear test. Freezing time for conditioned fear (**left panel**) and sensitized fear (**right panel**) in each group. ** $P<0.01$ vs. sham, # $P<0.05$ vs. MCAO.

cognitive deficits (i.e. reduced freezing time in both conditioned and sensitized fear) as compared to sham treatment ($P<0.01$), whereas HBO-PC abolished this effect ($P<0.05$ vs. MCAO). Meanwhile, there is also significant

difference between HBO+MCAO and sham in the freezing time in both conditioned and sensitized fear test ($P<0.05$). Suggesting the treatment of HBO-PC could not fully improve the cognitive deficits induced by MCAO.
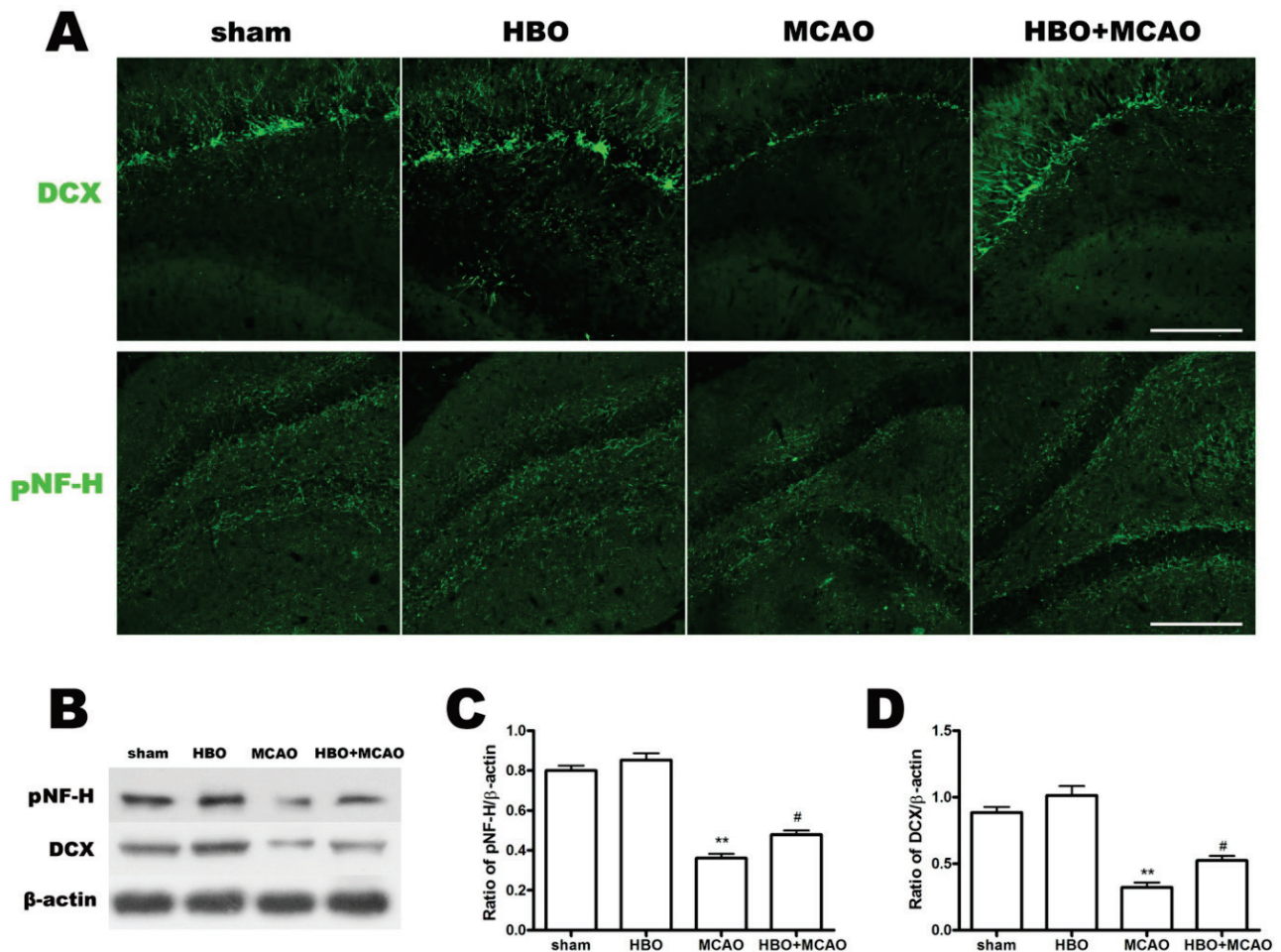

Fig. 3. HBO-PC increases pNF-H and DCX expression in the hippocampus of the ischemic brain. Representative photomicrographs of pNF-H and DCX expression in each group as determined by immunolabeling (**A**) and Western blot analysis (**B**). Quantitative analysis of pNF-H (**C**) and DCX (**D**) expression levels. ** $P<0.01$ vs. sham, # $P<0.05$ vs. MCAO. Bar: 200 μm.

HBO-PC restores pNF-H and DCX expression in the hippocampus following ischemia

The expression of pNF-H and DCX in the hippocampus differed between the four groups, as determined by immunohistochemistry (pNF-H: $F_{3,16}=90.05$, $P<0.01$ and DCX: $F_{3,16}=49.65$, $P<0.01$) and Western blotting (pNF-H: $F_{3,16}=88.27$, $P<0.01$ and DCX: $F_{3,16}=44.94$, $P<0.01$) (Fig. 3 and data not shown). A *post hoc* analysis revealed that pNF-H and DCX levels were significantly lower in the MCAO as compared to the sham group ($P<0.01$), effects that were mitigated by HBO-PC.

Sirt1 knockdown prevents HBO-PC-induced cognitive improvement

Conditioned fear ($F_{3,21}=9.204$, $P<0.01$) and sensitized fear ($F_{3,23}=10.65$, $P<0.01$) responses differed between the four groups (Fig. 4). siRNA-mediated knockdown of *Sirt1* abrogated the improvements in freezing time for both conditioned and sensitized fear induced by HBO-PC ($P<0.05$, HBO + siRNA-C vs. HBO + siRNA-Sirt1), whereas control siRNA treatment had no effect (i.e. there was no difference between siRNA-Sirt1 and HBO + siRNA-Sirt1 groups in terms of freezing time for both conditioned and sensitized fear; $P>0.05$).
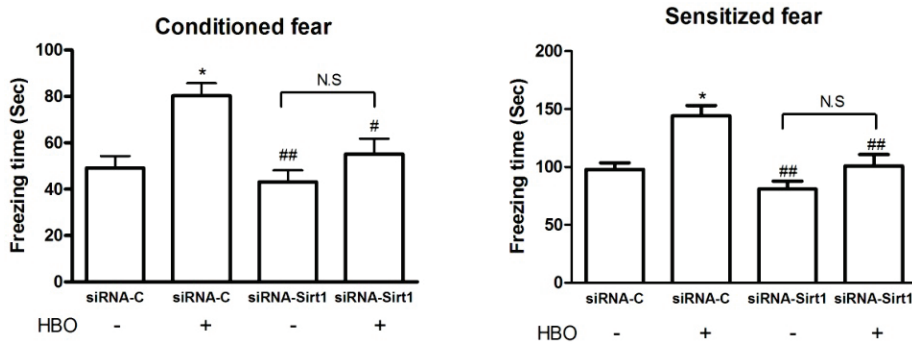
Fig. 4. Sirt1 knockdown alleviates the cognitive improvement associated with HBO-PC. Freezing time for conditioned fear (**left panel**) and sensitized fear (**right panel**) in each group. * $P<0.05$ vs. sham, [#] $P<0.05$ vs. HBO + siRNA-C, [##] $P<0.01$ vs. HBO + siRNA-C.

Sirt1 knockdown prevents HBO-PC-induced elevation of pNF-H and DCX expression in the hippocampus of ischemic hemispheres

Hippocampal expression of pNF-H and DCX differed significantly between the four groups, as determined by immunohistochemistry (pNF-H: $F_{3,16}$=84.57, $P<0.01$ and DCX: $F_{3,16}$=42.36, $P<0.01$) and Western blotting (pNF-H: $F_{3,16}$=14.99, $P<0.01$ and DCX:

$F_{3,16}$=11.97, $P<0.01$) (Fig. 5 and data not shown). A *post hoc* analysis revealed that pNF-H and DCX levels were higher in the HBO + siRNA-C as compared to the siRNA-C group ($P<0.05$), an effect that was abolished by *Sirt1* knockdown ($P<0.05$, HBO + siRNA-C vs. HBO + siRNA-Sirt1). There was no significant difference between siRNA-Sirt1 and HBO + siRNA-Sirt1 groups in terms of pNF-H and DCX expression ($P>0.05$).
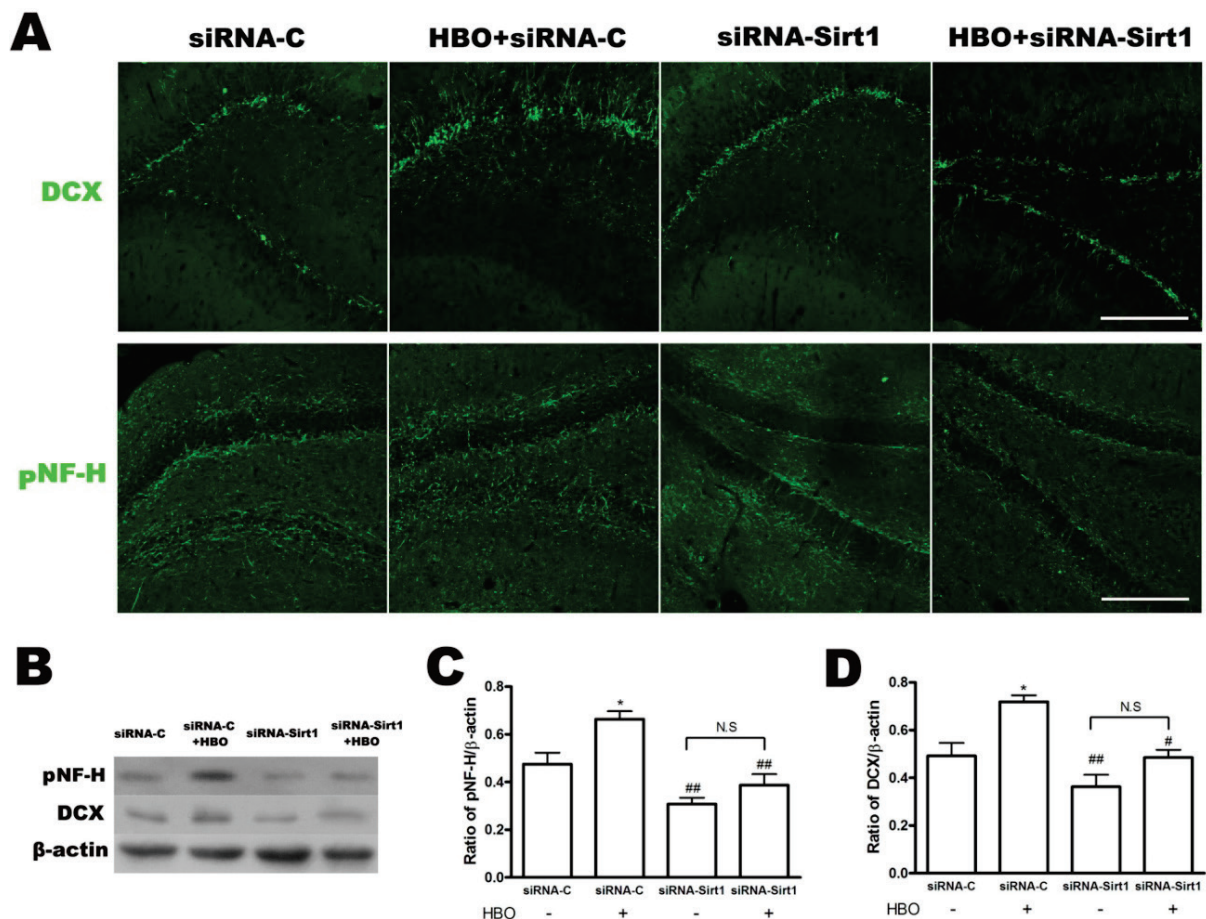
Fig. 5. Sirt1 knockdown inhibits pNF-H and DCX expression in the hippocampus of ischemic brain. Representative photomicrographs of pNF-H and DCX expression of each group as determined by immunolabeling (**A**) and Western blot analysis (**B**). Quantitative analysis of pNF-H (**C**) and DCX (**D**) expression levels. * $P<0.05$ vs. sham, [##] $P<0.01$ vs. HBO + siRNA-C. Bar: 200 μm.

Discussion

The present study investigated whether HBO-PC treatment can prevent cognitive deficits induced by focal cerebral ischemia, and whether Sirt1 is involved in this effect. The major findings were as follows: 1) HBO-PC 24 h before MCAO mitigated cognitive deficits; 2) DCX and pNF-H expression was upregulated in the ischemic brain after HBO-PC; and 3) *Sirt1* knockdown abrogated the benefits conferred by HBO-PC. These results indicate that application of HBO-PC and increasing Sirt1 levels can prevent neuronal injury caused by cerebral ischemia.

Cognitive deficits are a major factor affecting the life quality of ischemic stroke patients (Kwa *et al.* 1996). Ischemia can result in damage to the cerebral cortex (Saczynski *et al.* 2009) and hippocampus (Szabo *et al.* 2009, Tang *et al.* 2012). Reduction in the blood supply to the brain triggers various neuropathophysiological processes such as oxidative stress and inflammation that cause irreversible neuronal damage in non-ischemic regions (Butler *et al.* 2002, Block *et al.* 2005) as well as secondary damage that can affect cognition. HBO has demonstrated benefits in rodent models of ischemia induced by MCAO (Acka *et al.* 2007, Sun *et al.* 2011, Mu *et al.* 2013). HBO-PC has been similarly effective in human ischemia patients (Li *et al.* 2011b). However, it is not known whether HPO-PC can improve cognitive deficits induced by cerebral ischemia.

Transient and permanent cerebral ischemia occur *via* distinct mechanisms (Yang *et al.* 2001), with a more severe inflammatory response observed in the latter (Zhou *et al.* 2013). It has been reported that HBO-PC is effective in transient but not permanent ischemic injury models (Xiong *et al.* 2000). We therefore used a transient cerebral ischemia model (1 h MCAO) in the present study. MCAO not only causes infarctions in the ipsilateral cerebral cortex, but also secondary retrograde neurodegeneration in the hippocampus (Wang *et al.* 2004) that result in neurological deficits, including cognitive impairment and inhibition of synaptic transmission (Yang *et al.* 2015).

To assess contextual memory impairment, we trained rats on a fear-conditioned task and evaluated conditioned and sensitized fear responses following MCAO with or without HBO-PC. Consistent with a previous study (Yabuki *et al.* 2015), we found that MCAO reduced freezing time for both types of fear response, whereas HBO-PC mitigated this effect. Both the ventral and dorsal hippocampal regions are important

in the formation of conditioned fear memories (Esclassan *et al.* 2009); contextual fear conditioning requires gene transcription and protein synthesis in the hippocampus (Igaz *et al.* 2002). Moreover, neurogenesis and synaptic plasticity in the hippocampus is correlated with learning and memory (Drapeau *et al.* 2003). The mechanisms underlying secondary damage from ischemic stroke include retrograde axonal degeneration, neurotrophic dysregulation, and the upregulation of factors that inhibit neuronal growth (Yamashita *et al.* 2000).

To clarify the mechanistic basis for cognitive improvement by HBO-PC, we evaluated hippocampal expression of DCX and pNF-H. The former is a microtubule-associated protein in migrating neuroblasts that serves as a marker of immature neurons, whereas the latter is among the most highly phosphorylated proteins in the brain (Pant and Veeranna 1995); NF-H phosphorylation determines axonal caliber (Eyer and Peterson 1994), protects neurofilament proteins from proteolysis, and contributes to calcium buffering in axons (Krinks *et al.* 1988). Defects in NF-M/H phosphorylation in neurons can lead to blockage of axonal transport and cell death (Shea *et al.* 2004). We found that DCX and pNF-H levels were downregulated 14 days after MCAO; this reduction was alleviated by HBO-PC, suggesting that more neuroblasts were generated and neuroplasticity was increased by this treatment.

We also assessed conditioned and sensitized fear responses and hippocampal DCX and pNF-H expression upon *Sirt1* knockdown. Sirt1 is a class III histone deacetylase that has been linked to neuronal plasticity and protection (Kim *et al.* 2007, Gao *et al.* 2010). For instance, Sirt1 was shown to protect neurons against ischemic injury induced by resveratrol (Wan *et al.* 2016) and regulate senescence-associated proteins in response to oxidative stress (Mattagajasingh *et al.* 2007, Storz 2011). Our previous study demonstrated that Sirt1 mediates ischemic tolerance by HBO-PC (Yan *et al.* 2013) as well as long-lasting neuroprotection in rat brain (Xue *et al.* 2016). Here we showed that *Sirt1* knockdown blocked the improvement in memory and upregulation of DCX and pNF-H caused by HBO-PC, implying that Sirt1 contributes to HBO-PC-induced memory improvement by increasing neuroplasticity in the hippocampus.

Transient cerebral ischemia is known to stimulate neurogenesis in the dentate gyrus of adult rodents (Liu *et al.* 1998), which peaks on day 7 or 8 (Yagita *et al.* 2001, Takasawa *et al.* 2002); long-lasting neuroprotection can be observed for over 7 days after

reperfusion (Liu *et al.* 2012, Eady *et al.* 2014). Newborn neurons contribute to the formation of hippocampal-dependent memories (Bendel *et al.* 2005). However, the majority of these neurons are eliminated *via* apoptosis within several weeks (Kuhn *et al.* 2005); therefore, the therapeutic potential of neurogenesis induced by cerebral ischemia is low (Li *et al.* 2009). Stimulating neurogenesis and neuronal repair can help to overcome the low survival rate of neurons as a result of ischemia (Arvidsson *et al.* 2002).

In conclusion, our data suggest that HBO-PC promotes long-lasting improvements in memory following ischemia by preserving hippocampal neuroplasticity. These effects were mediated by Sirt1 activation. Further study is needed in order to clarify the detailed molecular mechanism of Sirt1 regulation in neurons and how this influences cognitive function.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

Abbreviations
DCX, doublecortin; HBO-PC, hyperbaric oxygen preconditioning; MCAO, middle cerebral artery occlusion; pNF-H, phosphorylated neurofilament heavy polypeptide; siRNA, short interfering RNA; Sirt1, sirtuin 1.

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