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Bone morphogenetic protein-7 (BMP-7) mediates ischemic preconditioning-induced ischemic tolerance via attenuating apoptosis in rat brain

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ABSTRACT

A mild cerebral ischemic insult, also known as ischemic preconditioning (IPC), confers transient tolerance to a subsequent ischemic challenge in the brain. This study was conducted to investigate whether bone morphogenetic protein-7 (BMP-7) is involved in neuroprotection elicited by IPC in a rat model of ischemia. Ischemic tolerance was induced in rats by IPC (15 min middle cerebral artery occlusion, MCAO) at 48 h before lethal ischemia (2 h MCAO). The present data showed that IPC increased BMP-7 mRNA and protein expression after 24 h reperfusion following ischemia in the brain. In rats of ischemia, IPC-induced reduction of cerebral infarct volume and improvement of neuronal morphology were attenuated when BMP-7 was inhibited either by antagonist noggin or short interfering RNA (siRNA) pre-treatment. Besides, cerebral IPC-induced up-regulation of B-cell lymphoma 2 (Bcl-2) and down-regulation of cleaved caspase-3 at 24 h after ischemia/reperfusion (I/R) injury were reversed via inhibition of BMP-7. These findings indicate that BMP-7 mediates IPC-induced tolerance to cerebral I/R, probably through inhibition of apoptosis.

1. Introduction

Ischemic stroke remains a vexing public health problem. Nevertheless the corresponding pharmacological treatments are either ineffective or confounded by adverse effects [1]. Endogenous mechanisms by which the brain protects itself against noxious stimuli and recovers from damage are thus being studied [2]. Of note, ample evidence has proved that, in the brain, ischemic preconditioning (IPC) that refers to a sufficient but non-injurious ischemic insult provides potent protection against a subsequent, otherwise lethal ischemia [3,4].

Bone morphogenetic proteins (BMPs) are originally identified as regulators of cartilage and bone formation [5,6]. However, more recent work indicates their effects are not limited in the skeletal system. They are found to regulate the growth, differentiation, chemotaxis and apoptosis of various cell types, including epithelial, mesenchymal, haematopoietic and neuronal cells [5,7]. Of these BMPs, BMP-7 is believed to exert unique protective and regenerative effects on brain injury in animals. BMP-7 expresses in many neuronal tissues, such as hippocampus, cortex and cerebellum [8], and is able to promote DNA synthesis and astroglial cell differentiation in the midbrain floor of the rats [9]. Notably, in BMP-$7^{+/-}$ mice, the post-stroke recovery of locomotor activity is impaired as compared with wild-type littermate controls [8]. Moreover, elevating BMP-7 improves the recovery from ischemic brain damage in animals. In neonatal rats, intra-peritoneal administration of BMP-7 before general hypoxia reduces brain infarction volume and mortality [10]. Likewise, intracerebral injection of BMP-7 prevents adult rats from middle cerebral artery occlusion (MCAO)-caused cerebral infarction [11]. Although these earlier studies indicate a crucial role of BMP-7 in ischemic injury in rodents, its exact mechanism still remains to be fully elucidated. Interestingly, a recent study indicates that exogenous BMP-7 probably duplicates IPC effects to ameliorate the subsequent ischemia/reperfusion (I/R) injury in the intestine and liver [12]. These findings lead us to examine whether BMP-7 contributes to IPC-induced ischemic tolerance in the brain.

In the present study, we used a rat model of ischemic tolerance induced by sublethal MCAO-preconditioning to determine the effect of BMP-7 on cerebral ischemic injury.

2. Methods

2.1. Animal model and experimental grouping

The experimental protocol has been approved by the ethics committee of China Medical University, and it conforms to the

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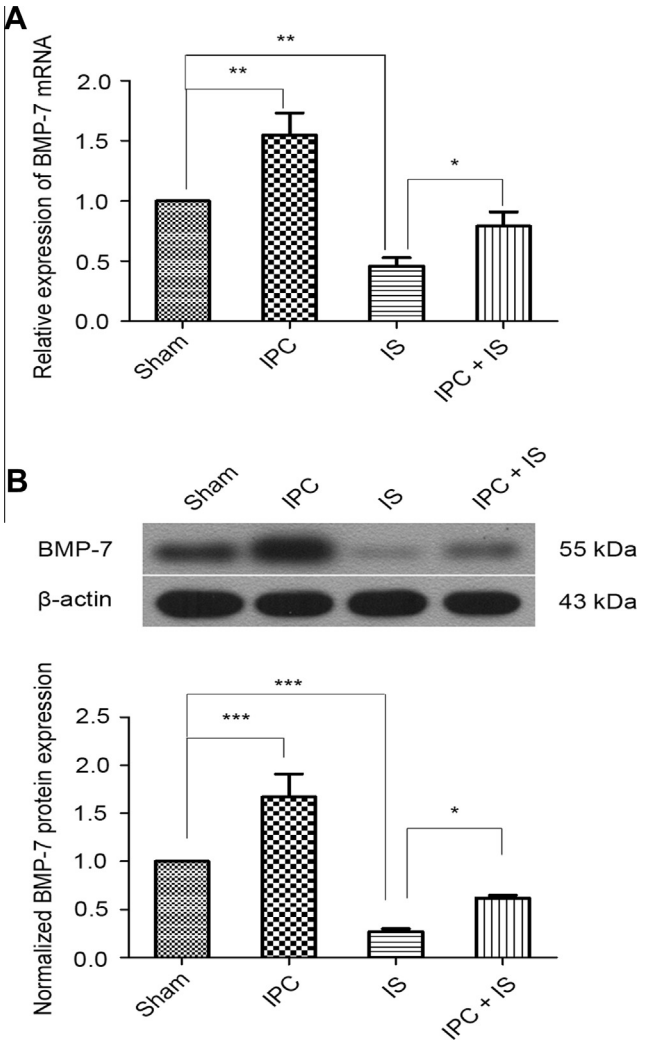

Fig. 1. IPC up-regulates BMP-7 mRNA and protein in rat brain. (A) The expressions of BMP-7 mRNA ($n = 4$ per group) and (B) Western blot bands and quantitative evaluation of BMP-7 protein ($n = 5$ per group) at 24 h after reperfusion. β-Actin served as endogenous control. Data were presented as mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. IPC, ischemic preconditioning; IS, ischemia.

provisions of the declaration of Helsinki in 1995 (as revised in Edinburgh 2000). Male Wistar rats (weighing from 280 to 320 g) were housed at a constant room temperature (20–22 °C) and humidity (50–60%) with a 12 h light and 12 h dark cycle, and freely fed a standard diet and tap water.

According to previous protocols [11,13,14], ischemic tolerance was induced in anesthetized rats by a mild forebrain ischemia (MCAO for 15 min) followed by a 48 h recovery. These animals were then subjected to lethal ischemic insult (MCAO for 2 h). Then, rats were sacrificed for the following study.

2.1.1. Part I: The basal expression of BMP-7 after ischemic insults in rat brain

Rats were allocated randomly into four groups based on the IPC stimulus: (1) Sham, rats were subjected to sham operation; (2) IPC, rats were subjected to MCAO for 15 min. Forty-eight hours later, rats were subjected to lethal ischemic insult: (3) ischemia (IS), normal rats received 2 h MCAO; (4) IPC + IS, rats of IPC received 2 h MCAO.

To investigate the expression profile of BMP-7 at different ischemic conditions, rats from the above four groups were sacrificed at 24 h after the last treatments, and the brain tissues were then subjected to detect the mRNA ($n = 4$ per group) and protein ($n = 5$ per group) levels of BMP-7.

2.1.2. Part II: The effect of BMP-7 antagonist noggin on the protective effect of IPC in rat brain

Normal saline with or without BMP-7 antagonist noggin was injected into rats 30 min before IPC: (5) IPC + IS + Noggin; (6) IPC + IS + Vehicle. Brain samples from Group 3 to Group 6 were subjected to infarction assessment ($n = 6$ per group) at 24 h and 7 d after I/R injury, and Western blot analysis ($n = 5$ per group) for B-cell lymphoma 2 (Bcl-2) and cleaved caspase-3 at 24 h after reperfusion. Moreover, neuropathological evaluation was performed in rat brains from Group1 to Group 6 at the end of the experiment ($n = 5$ per group). Sham operated rats received noggin treatment served as control: (7) Sham + Noggin ($n = 5$).

2.1.3. Part III: The effect of BMP-7 short interfering RNA on the protective effect of IPC in rat brain

Based on earlier reported protocols [15], *in vivo* injection of BMP-7 short interfering RNA (siRNA) or control siRNA was performed in rats 30 min before IPC: (8) IPC + IS + siRNA-BMP-7; (9) IPC + IS + siRNA-Control. Like in Part II, infarction assessment ($n = 6$ per group) and Western blot analysis ($n = 5$ per group) were performed in rats from these two groups and that from Group 3 to Group 4.

2.2. Quantitative real-time PCR

Total RNAs from brain tissues were extracted using RNA simple total RNA kit (TianGen, Beijing, China), and cDNAs were synthesized using Super M-MLV reverse transcriptase kit (BioTeke, Beijing, China). SYBR Green (Solarbio, Beijing, China) was used for real-time PCR on Exicycler™ 96 (Bioneer, Daejeon, Korea), and the mRNA expression levels of BMP-7 were quantified via $2^{-\Delta\Delta CT}$ [16]. Analytical data were normalized to the mRNA expression level of endogenous control β-actin. The primers used in quantitative real-time PCR were BMP-7 (forward 5′ ATCCCCAATGTCTCACCACCTA 3′ and reverse 5′ AAGTATGCTGCTTATCAACCACG 3′), and β-actin (forward 5′ GGAGATTACTGCCCTGGCTCCTAGC 3′ and reverse 5′ GGCCGGACTCATCGTACTCCTGCTT 3′).

2.3. Western blot analysis

Protein samples from brain tissues were subjected to Western blot analysis using polyclonal antibodies for BMP-7 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bcl-2 (Boster, Wuhan, China) and cleaved caspase-3 (Bioss, Beijing, China). Protein blots were detected using the ECL-Plus Western blotting detection system.

2.4. Infarction assessment

At 24 h and 7 d after I/R, rats were decapitated and the brain tissues were then removed, immersed in cold saline for 5 min, and sliced into 2.0-mm-thick sections for triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO, USA) assessment. The percentage of the infarct volume was determined by indirect measurement based on the non-infracted cortex volume according to a previous report [17].

2.5. Neuropathological evaluation

The sections for neuropathological evaluation were stained with thionin (Solarbio, Beijing, China). Neuropathological evaluation of hippocampal CA1 subfield was subjected to determination of the delayed neuron death by histological grade (HG) [18] and neuronal density (ND) [19].

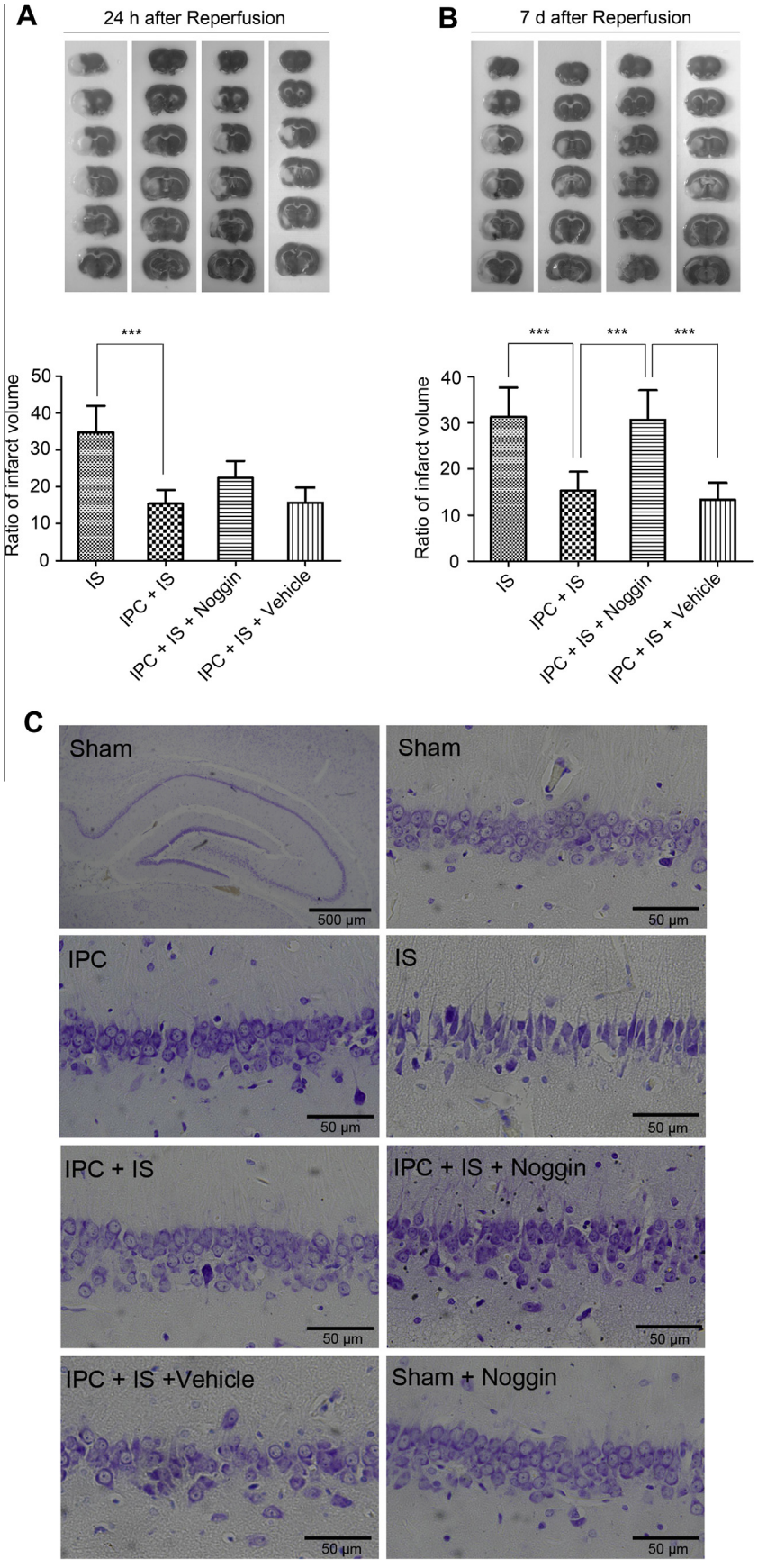

Fig. 2. Inhibition of BMP-7 via its antagonist noggin reverses the protection of IPC in rat brain. Representative 2,3,5-triphenyltetrazolium chloride (TTC)-stained thick brain sections and quantitative evaluation of the infarction volume at 24 h (A) and 7 d (B) after ischemia/reperfusion (I/R) ($n = 6$ per group). Representative thionin stained neurons of hippocampal in CA1 subfield ($n = 5$ per group) at 7 d after I/R (C). Data were presented as mean ± standard deviation (SD). $^{***}P < 0.001$. IPC, ischemic preconditioning; IS, ischemia.

Table 1
The histological grade (HG) and pyramidal neuronal density (ND) of the hippocampal CA1 region in each of the groups.

Group	Histological grade				ND[a]
	0∼	I∼	II∼	III	
Sham	6	–	–	–	192.83 ± 19.17
IPC	5	1	–	–	185.00 ± 23.67
IS	–	–	1	5	33.50 ± 8.50***
IPC + IS	1	3	2	–	121.17 ± 18.50$
IPC + IS + Noggin	–	2	3	1	72.17 ± 13.50#
IPC + IS + Vehicle	–	4	2	–	112.33 ± 13.33
Sham + Noggin	6	–	–	–	195.33 ± 18.33

[a] Data are indicated as mean ± SD.
*** $P < 0.001$ compared with IPC + IS.
$ $P < 0.001$ compared with IPC + IS + Noggin.
# $P < 0.01$ compared with IPC + IS + Vehicle. Histological grades: Grade 0, no neuron death; Grade I, scattered single neuron death; Grade II, mass neuron death; Grade III, almost complete neuron death.

Animals were decapitated and 3-mm-thick brain slices including the bilateral dorsal hippocampus were excised coronally and fixed in 4% paraformaldehyde for 6 h. These slices were dehydrated with alcohol, cleared with xylene and stained with thionin. The thionin-stained brain tissues were then subjected to HG and ND assessments. HG was divided into four grades and the standard is as follows: grade 0, no neuron death; grade I, scattered single neuron death; grade II, mass neuron death; grade III, almost complete neuron death. ND was determined by counting the number of surviving pyramidal neurons with intact cell membrane, full nucleus, and clear nucleolus within 1 mm linear length of the CA1. The average number of pyramidal neurons in six areas of the hippocampal CA1 subfield was calculated as the ND value.

2.6. Injection of BMP-7 antagonist noggin in vivo

BMP-7 antagonist noggin (R&D systems, MPLS, MN, USA) was first dissolved in DMSO and then diluted in normal saline (the final DMSO concentration <2%). Intracerebroventricular injection of normal saline with or without 2 μg noggin was performed 30 min before the onset of MCAO preconditioning.

2.7. Administration of siRNA in vivo

The duplexed RNA oligonucleotides for rat BMP-7 (5′ GCCGAGUUCAGGAUCUAUATT3′ and 5′ UAUAGAUCCUGAACUC GGCTT 3′) were designed and synthesized by GenePharma (Shanghai, China). We performed *in vivo* siRNA injection (5 μg) in rat brain according to the methods described previously [15,20,21]. Scramble siRNA served as control.

2.8. Statistical analysis

All data were presented as the mean ± standard deviation (SD). Statistical analysis was carried out by ANOVA, followed by *T* test for multiple comparisons using the SPSS 17.0 program. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. IPC up-regulates BMP-7 mRNA and protein expression in rat brain

As compared with sham group, the mRNA level of BMP-7 significantly increased in IPC treated rats ($P < 0.01$), whereas decreased in IS treated rats ($P < 0.01$). IPC partially reversed IS-induced down-regulation of BMP-7 mRNA expression (Fig. 1A). Likewise, Western blot results indicated a corresponding changing pattern of BMP-7 protein at different ischemic conditions (Fig. 1B). These results indicate that IPC induces the expression elevation of BMP-7 in rat brain.

3.2. Noggin attenuates IPC-induced reduction of cerebral infarct volume and improvement of neuronal morphology after I/R in rats

Infarct volume is one of the common indexes for assessing the extent of ischemic brain injury following cerebral ischemia [1,2]. Here we found that ligation of MCA for 2 h and reperfusion for 24 h or 7 d resulted in a clear-cut infarction of the cortex in rats, whereas the mild IPC significantly reduced this alteration (Fig. 2A and B), corresponding to the earlier reported work [22]. Noggin injection barely affected IPC-induced attenuation in head infarction at 24 h after reperfusion (Fig. 2A), but it abolished IPC-induced therapeutic changes after 7 d reperfusion (Fig. 2B).

Moreover, we also examined the morphologic changes of the neurons in hippocampal CA1 subfield after diverse ischemic treatments. The pyramidal neurons of sham or noggin treated sham rats remained untouched. These neurons showed normal clear cell outline, compact and abundant cytoplasm (Fig. 2C). No obvious neuronal damage was observed in the brain from IPC group (Fig. 2C). On the other hand, IS induced evident neuronal degeneration, and this pathological change was markedly attenuated by IPC (Fig. 2C). However, noggin treatment abolished this neuroprotective action of IPC in ischemic injury (Fig. 2C). In addition, the corresponding HG and ND results supported the above histological results (Table 1). The above results illustrate that the elevated BMP-7 may be involved in the neuroprotective action of IPC-induced ischemic tolerance.

3.3. Downregulation of BMP-7 by siRNA diminishes IPC-induced ischemic tolerance in rat brain

We first examined the knockdown efficiency of BMP-7 specific siRNA in rat brain. As expected, constant decreased protein levels of BMP-7 were observed at 1, 3, 5, and 7 d after injection of BMP-7 siRNA, when compared with the value before the onset of siRNA injection (Fig. 3A). The control siRNA did not alter the expression of BMP-7 during the experimental process (Fig. 3B). Like in noggin-treated animals, knocking down BMP-7 expression by its siRNA attenuated IPC-induced self-protection of the brain (Fig. 3C and D).

3.4. BMP-7. Participates in IPC-induced ischemic tolerance by attenuating apoptosis in rat brain

We continued to explore the regulatory role of BMP-7 in IPC with an emphasis on neuronal apoptosis. We noted that the pretreatment of noggin or BMP-7 siRNA reversed IPC-induced up-regulation of Bcl-2 and down-regulation of cleaved caspase-3 (Fig. 4). Such result suggests BMP-7 contributes to IPC-induced ischemic tolerance by inhibiting apoptosis in rat brain.

4. Discussion

Recent work has delineated a role of BMP-7 in IPC in various organs. Earlier microarray analyses conducted in rodents elucidate that IPC induces a significant up-regulation of endogenous BMP-7 in kidney [23] and intestine [24]. Our data supported such prior results by showing that the mRNA and protein levels of BMP-7 increased after an initial insult of minor ischemia (IPC) in rat brain. On the other hand, we found that the lethal ischemia (2 h MCAO) resulted in a sharp decrease in BMP-7 expression, and this

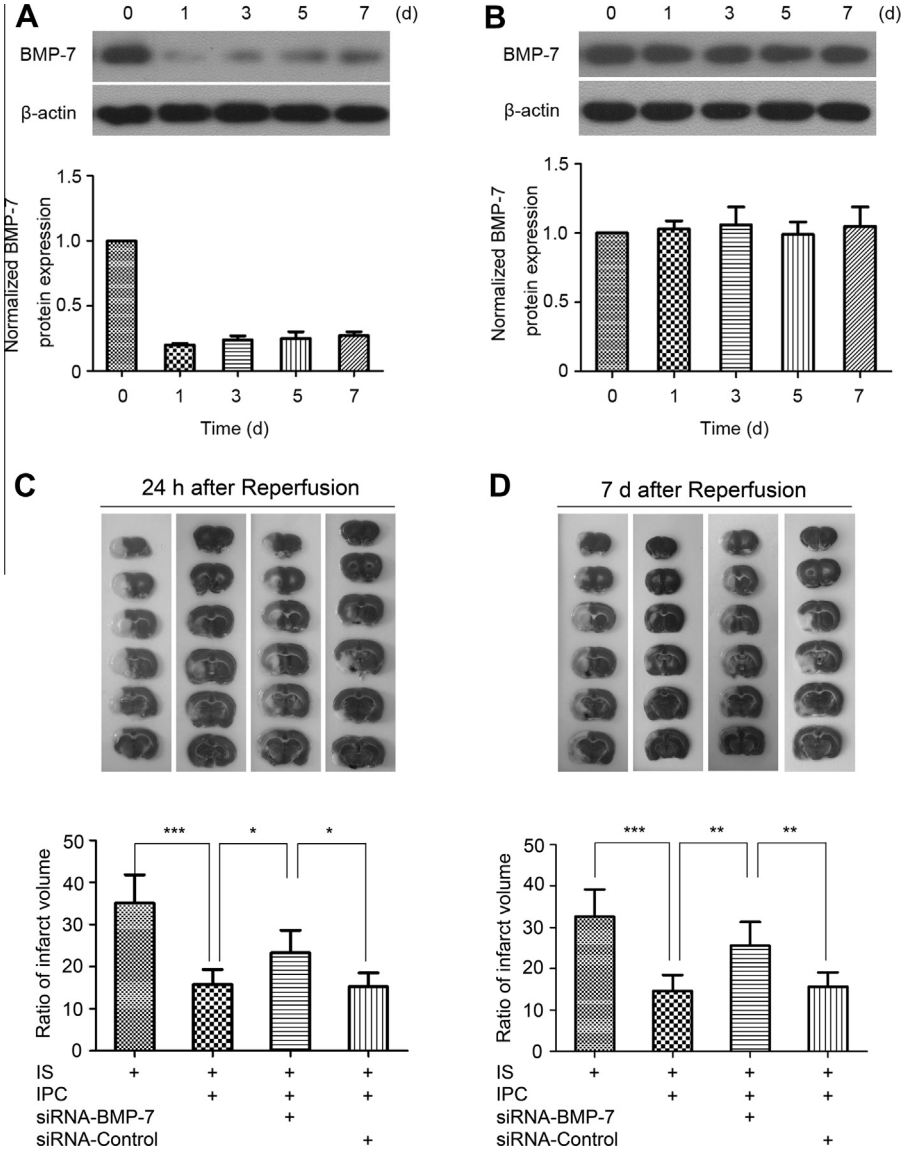

Fig. 3. Knockdown of BMP-7 by short interfering RNA (siRNA) reverses the protective effect of IPC on rat brain. The protein expression of BMP-7 was detected by Western blot in rats from day 1 to day 7 after (A) BMP-7 siRNA or (B) control siRNA injection ($n = 25$ per group). Representative 2,3,5-triphenyltetrazolium chloride (TTC)-stained thick brain sections and quantitative evaluation of the infarction volume at 24 h (C) and 7 d (D) after ischemia/reperfusion (I/R) ($n = 6$ per group). Data were presented as mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. IPC, ischemic preconditioning; IS, ischemia.

reduction was attenuated by mild IPC markedly. In contrast to the findings presented here, Chang et al. [25] have showed that severe ischemic insult enhances BMP-7 expression. One possible explanation for this disagreement may arise from different experimental subjects and protocols. They conducted experiments on Sprague–Dawley rats, and only obtained the BMP-7 mRNA expression pattern at 8 h after I/R, while we obtained both mRNA and protein data of BMP-7 on Wistar rats at 24 h after I/R. Taken together, we hypothesize that IPC may work through up-regulation of BMP-7 to protect against ischemia in animal brain.

Radhakrishnan et al. [12] have proposed a hypothesis that the exogenous BMP-7 replicates the effects seen with IPC in the intestine and liver after I/R in rats. As an indirect support to such hypothesis, we showed that inhibition of the endogenous BMP-7 expression via noggin abolished IPC-induced protection in the brain. Of note, after stroke, there is no difference in total, cortical, or striatal infarct volume between vehicle-treated animals and those receiving post-injury injection of BMP-7 [26]. On the other hand, pretreatment with exogenous BMP-7 at 24 h before stroke significantly reduces the volume of infarction in the cortex [11]. These two prior reported studies indicate that only introduction of BMP-7 before severe ischemia may attenuate the following cerebral damage, indicating BMP-7 contributes to IPC-induced ischemic tolerance in animal brain. In addition, we and others [27,28] have proved IPC happened before ischemia evokes neuroprotective mechanisms in the brain. However, suppression of the increased BMP-7 via noggin also blocked this protective effect of IPC in neurons. Since noggin is a low affinity antagonist to BMP-7 [29,30], we used the specific siRNA to exclusively knock down BMP-7 expression in rat brain. Like in noggin treated rats, the IPC effect almost disappeared in rats subjected to treatment with BMP-7 siRNA. Such results once again demonstrated the pivotal role of BMP-7 in protection of IPC in cerebral ischemic lesion.

Many studies have demonstrated that ischemia induces cell degradation and apoptosis accompanied by decreased Bcl-2 [31] and increased cleaved caspase-3 in the ischemic core [32]. Because the severity of cerebral ischemia depends mainly on neuronal survival or death [28], we investigated whether the neuroprotective

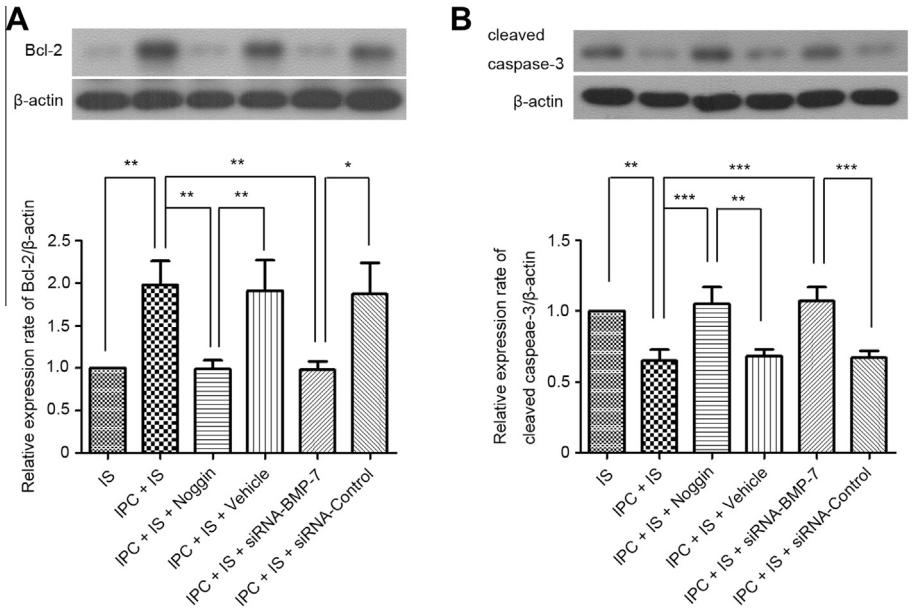

Fig. 4. Modulation of Bcl-2 and cleaved caspase-3 by IPC and BMP-7 in rat brain. Representative Western blot bands and quantitative evaluation of Bcl-2 (A) and cleaved caspase-3 (B) expression. β-actin served as endogenous control. Data were presented as mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. IPC, ischemic preconditioning; IS, ischemia.

mechanism of IPC mediated by BMP-7 expression was associated with inhibition of apoptosis, with an emphasis on Bcl-2 and caspase-3 singling. Shimizu et al. [33] have reported that focal ischemia increases Bcl-2 expression in the rat brain, and contrarily, neutralizing the effect of Bcl-2 via its antisense accelerates the lesion induced by subsequent global ischemia. Moreover, Han et al. [34] have showed that the hypoxia–ischemia injury to the developing brain leads to caspase-3 activation, and blocking this activation inhibits the subsequent apoptosis. These two previous researches suggest either up-regulation of anti-apoptotic protein Bcl-2 or down-regulation of pro-apoptotic protein cleaved caspase-3 brings in robust protection of cells against death in ischemic lesion of brain. In this study, we have found that inhibition of BMP-7 by its antagonist or siRNA can suppress Bcl-2 and promote cleaved caspase-3, implying that the inhibition of apoptosis is involved in neuroprotection of the interplay between IPC and BMP-7.

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