

Original Article

Mild hypothermia combined with isoflurane post-treatment alleviates cerebral ischemia reperfusion injury in rats through inhibition of STAT3 activation

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Received July 25, 2016; Accepted September 18, 2016; Epub December 15, 2016; Published December 30, 2016

Abstract: We conducted this study to assess the effect of mild hypothermia combined with isoflurane on cerebral ischemia reperfusion injury and p-STAT3 expression in rats and its possible mechanism. Fifty healthy adult male Sprague-Dawley rats were randomly divided into 5 groups: Control group (Ctrl group), Model group (I/R group), Mild hypothermia (MH group), Isoflurane group (Iso group) and Mild hypothermia combined with isoflurane group (MH+Iso group). After 24 h reperfusion, neurological deficit scores were evaluated. Then, rats were sacrificed and five of them were used to detect infarct volume by TTC staining method. The other five served to measure mRNA levels of STAT3, IL-6, GFAP and Iba1 in cerebral ischemia cortex by qRT-PCR and protein levels of p-STAT3, STAT3, p-JAK2, JAK2, GFAP and Iba1 by Western blot and IL-6 by ELISA method. Compared with I/R group, neurologic impairment score, volume of cerebral infarction and the value of p-STAT3/t-STAT3 in MH, Iso and MH+Iso groups were obviously decreased ($P < 0.01$). The express levels of IL-6, GFAP, Iba1 and phosphorylation level of JAK2 in post-treatment (MH, Iso and MH+Iso) groups were significantly lower than that in I/R group. Among the post-treatment groups, the neurologic impairment score was the lowest, volume of cerebral infarction was minimum and the decreasing levels of p-STAT3/t-STAT3, IL-6, p-JAK2/JAK2, GFAP and Iba1 were maximum ($P < 0.05$) in MH+Iso group ($P < 0.05$). In conclusion, mild hypothermia combined with isoflurane post-treatment can significantly reduce rat cerebral ischemia reperfusion injury, the effect is better than that of mild hypothermia or isoflurane post-treatment alone. The mechanism may be that mild hypothermia combined with isoflurane post-treatment inhibited JAK2/p-STAT3 pathway in astrocytes or microglia, thereby inhibiting excessive activation of microglia and proliferation of astrocytes, and eventually they protected ischemic cerebral neurons from I/R injury.

Keywords: Cerebral ischemia reperfusion injury, mild hypothermia, isoflurane, p-STAT3

Introduction

In spite of the fact that ischemic stroke is still one of leading causes of death and long-term disability worldwide, few curative therapeutic strategies are available for patients exposed to cerebral ischemia [1]. Restoration of blood flow to the ischemic brain with tissue plasminogen activator (tPA) treatment is currently the effective way to protect the brain against ischemic brain damage. However, reperfusion and administration of tPA are strictly limited by the narrow therapeutic time window [2]. If exceeds

the time window, the risk rate of hemorrhagic transformation and fatal edema induced by cerebral ischemia/reperfusion (I/R) injury would increase paradoxically [3].

At present, the pathogenesis of cerebral I/R injury is not completely clear yet. JAK-STAT signaling pathway is stimulated by cytokines, and is involved in a variety of important biological events, such as cell proliferation, differentiation, apoptosis and immune modification. Accumulating evidence indicates that JAK-STAT signaling pathway may play an important

role in cerebral I/R injury. It was found that focal cerebral ischemia would activate JAK-STAT signaling pathway in the post-ischemic brain, predominantly in the macrophages/microglia, and treatment with AG490 (a JAK2 phosphorylation inhibitor) or siRNA specific for STAT3 (an inhibitor for STAT3 mRNA expression and phosphorylation) significantly decreased the infarct volume, rate of apoptosis in cells and neurological deficits induced by cerebral I/R [4]. Tong-Chun Wen *et al.* found that the number of p-STAT3-positive neurons was increased in the peripheral part of the ischemic area at 24 h after ischemia, and p-STAT3-positive neurons were at different stages of degeneration, suggesting that the activation of STAT3 after cerebral ischemia may contribute to ischemia-induced neuron death [5]. Shigeaki Suzuki *et al.* also found that phosphorylated STAT3 was detected in neurons after 3.5 h of reperfusion, peaked at 24 h, and then decreased gradually [6].

At present, mild hypothermia (MH) may be the single most effective neuroprotective strategies, which has been shown to have neuroprotective function in cerebral ischemia both experimentally and clinically [7-10]. However, the mechanisms underlying hypothermic protection in cerebral I/R remain to be further elucidated. It was found that hypothermic treatment could significantly reduce the secondary injury to brain after ischemia through attenuation of STAT3 action [11]. Isoflurane (Iso) is a commonly used volatile anesthetic. It has been reported that pretreatment with Iso could provide ischemic tolerance in brain [12, 13]. Multiple mechanisms have been reported to explain the beneficial role of Iso; however, the exact underlying mechanisms of protection are still not clear.

We hypothesized that combination of mild MH and Iso might improve the cerebral protective effect in cerebral I/R injury, and thus improve clinical outcomes in patients suffering from ischemic stroke. The present study aimed to investigate the improvement effect of pretreatment with MH in combination with Iso on cerebral I/R injury in rat model and the underlying mechanisms, mainly focusing on the role of JAK-STAT signaling pathway in cerebral I/R injury mechanism and therapeutics by MH and Iso pretreatments through quantifying the levels of p-STAT3, STAT3, p-JAK2, JAK2, IL-6, GFAP and Iba1 in rat cerebral ischemia cortex.

Materials and methods

Animals and groups

Fifty SPF male Sprague-Dawley (SD) rats, 7-8 weeks old, weighing 250-300 g, were purchased from Shanghai Slrc Laboratory Animal Co. Ltd. (number of animal license SCXK 2012-0002; animal certification number 2015-000506055, 2015000507955). The rats were raised as usual under a regular 12 h light/dark cycle, ingest and drinking freely. All rats were randomized into control group (Ctrl group), model group (I/R group), mild hypothermia (MH group), Isoflurane group (Iso group) and mild hypothermia combined with isoflurane group (MH+Iso group), with 10 rats in each. All the experimental procedures were conducted in conformity with the guidance suggestions for the care and use laboratory animals formulated by the Ministry of Science and Technology of the People's Republic of China.

Establishment of I/R model and treatments

Rats were starved for 12 h prior to surgery and water was taken freely. Rats in I/R group were made into models of middle cerebral artery occlusion (MCAO) by thread embolism method. Briefly, after the rat was anesthetized with 10% chloral hydrate (3.5 mL/kg, *i.p.*), a cervical median incision was made. The left common carotid artery (CCA), the external carotid artery (ECA) and the internal carotid artery (ICA) were exposed under sterile conditions; then the CCA and ECA were ligated. A small incision in the inferior side of the bifurcation of the CCA was cut with corneal scissors. Then, a silicone-coated intraluminal filament was advanced into the ICA 18 to 20 mm until mild resistance indicated that the tip was lodged in the anterior cerebral artery and thus blocked blood flow to the middle cerebral artery (MCA). The models realized reperfusion after 2 hours MCAO by removing the filament. The rats were breathing spontaneously and rectal temperature was maintained at 37.0-37.5°C throughout the procedure. The control group rats were subjected to the same surgical procedure but the MCA was not occluded. Rats in MH and MH+Iso groups were cooled down their head temperatures using homemade ice packs, immediately following the initiation of ischemia, which all reached 33.6±0.5°C. Rat heads were maintained at this temperature until the completion of reperfu-

Cerebral ischemia-reperfusion injury protection

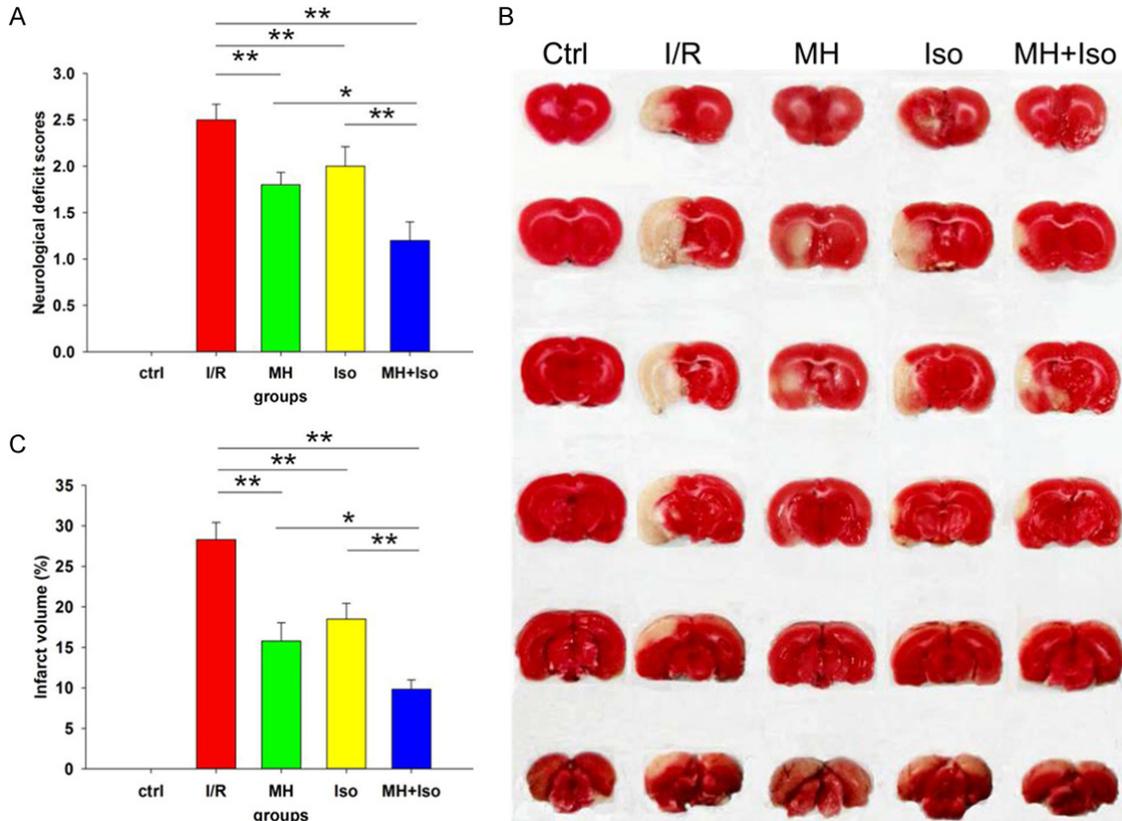


Figure 1. Neurological deficit score (A) and cerebral infarct volume (B, C) in each group after 24 h reperfusion, Data are expressed as mean \pm SD (n=5), *P < 0.05, **P < 0.01.

sion. Rats in Iso and MH+Iso groups were treated in 2% Isoflurane for 1 h after the completion of reperfusion.

Neurological deficit score

Evaluation of the neurological deficit in rats at 24 h of reperfusion was based on the method of Longa *et al.* [14] with modifications. Briefly, the motor findings were scored on a five-point scale: 0, no observable deficit, rat extend the two forepaws fully when lifted by snatching the tail; 1, forelimb flexion, tail snatching experiment are positive; 2, rotation to the left; 3, Falling to the right; 4, unable to ambulate to the left or coma. Rats, scored 1 to 4 were regarded as success cerebral I/R injury model. The rats were eliminated when the following occurs: massive hemorrhage, labored breathing, sub-arachnoid hemorrhage and premature death.

Cerebral infarct volume assay

After neurological assessment, rats under anesthesia were decapitated, and the brain

was removed quickly. Each brain was frozen at -80°C for 5-10 min before cut into coronal sections at 2 mm with brain slot. Then, the sections were stained with 1% TTC (pH 7.4) at 37°C , in darkness and fixed in 4% paraformaldehyde solution for 24 h. The size of the infarct area (white) was quantified with Image Pro plus 6.0. The total infarct volume was calculated with the following formula: the total infarct volume = the infarct areas \times section thickness/ the volume of the left hemisphere \times 100%.

qRT-PCR

The rat was anesthetized with 10% chloral hydrate (3.5 mL/kg, *i.p.*) after 24 h of reperfusion and cerebral ischemic cortexes were obtained. The mRNA levels of STAT3, IL-6, GFAP and Iba1 were measured in 5 ischemic cortexes samples from each group. Total RNA was isolated using Trizol RNA isolation system (Invitrogen, U.S.) according to the manufacturer's instructions and an aliquot of 500 ng total RNA was used for first strand cDNA synthesis

Cerebral ischemia-reperfusion injury protection

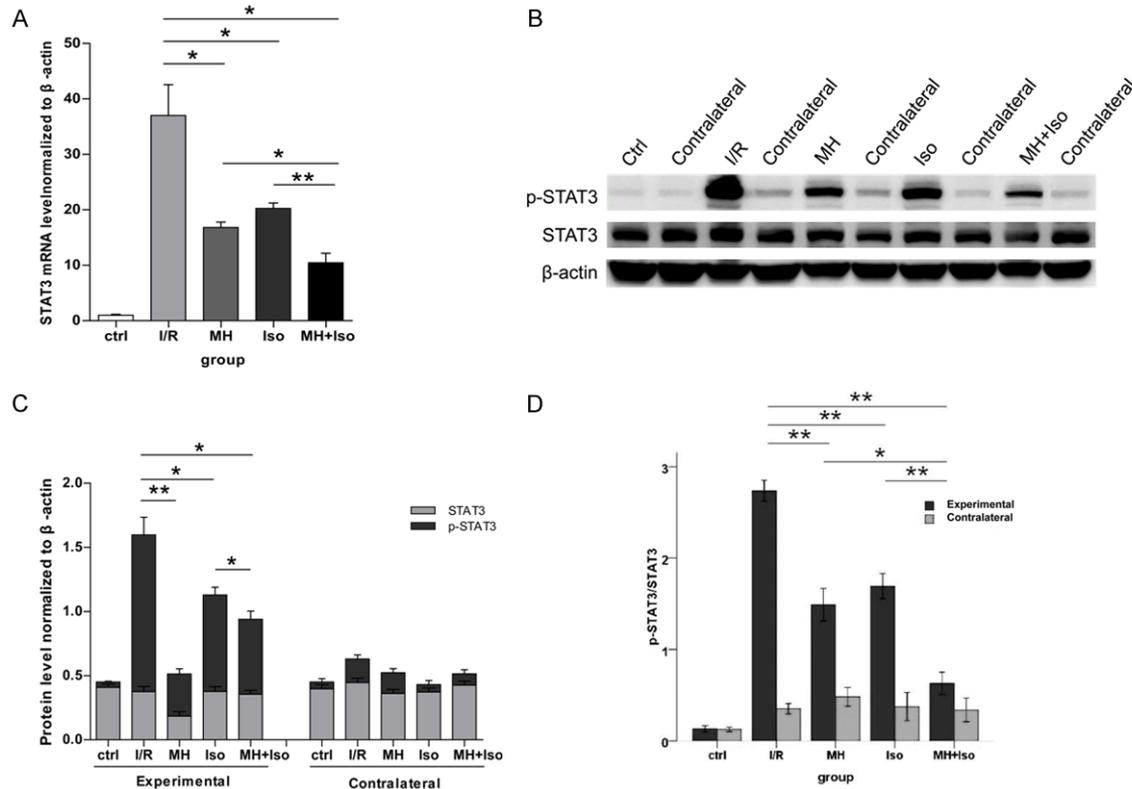


Figure 2. The mRNA level of STAT3 (A), protein levels of STAT3 and p-STAT3 (B, C) and STAT3 phosphorylation level (D). Data are expressed as mean \pm SD (n=5), *P < 0.05, **P < 0.01.

using PrimeScript™ RT reagent Kit (TaKaRa, Japan). Target gene or control β -actin sequences were then PCR amplified from cDNA. The expression of STAT3, IL-6, GFAP and Iba1 relative to β -actin was measured through qRT-PCR.

The PCR primers (5'→3') were described as follows:

STAT3: F: TCGGAAAGTATTGTCGCCCC; R: GACATCGGCAGGTCAATGGT. IL-6: F: CACTTCACAAGTCGGAGGCT; R: TCTGACAGTGCATCATCGCT. GFAP: F: AAATTGCTGGAGGGCGAAGA; R: CCGCATCTCACCCTCTTTA. Iba1: F: TTAGAGAGGTGTCAGTGGC; R: CTTTTCCCATGCTGCTGTCA. β -actin: F: CCACCCGCGAGTACAACCTT; R: GTCCTTCTGACCATACCCAC.

Western blotting

After 24 h reperfusion, the cerebral ischemic cortexes was removed and weighed before homogenized on ice in cell lysis buffer containing 1 mmol/L complete proteinase inhibitor

cocktail, and then the superior solution was got after centrifugation at 12,000 g at 4°C for 5 min. Protein concentration was determined by the BCA assay kit. The protein concentrations of the samples were adjusted to the same with loading sample buffer before the samples were heated at 100°C for 5 min. Then the samples were loaded and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (concentration gel 80 V and separation gel 120 V) and then transferred to a PVDF membrane (150 mA, 2 h). The membrane was blocked with 5% BSA for 2 h and then incubated for 24 h at 4°C with primary antibodies to p-STAT3 (1/200000, Abcam, ab76315), STAT3 (1/5000, Abcam, ab119352), p-JAK2 (1:1000, CST, #3776), JAK2 (1:1000, CST, #3230), GFAP (1:1000, CST, #12389) and Iba1 (1:500, Abcam, ab15690). After washing three times with TBST for 10 min, the membrane was incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The quantitative protein band density was detected and assayed by the Quantity One system.

Cerebral ischemia-reperfusion injury protection

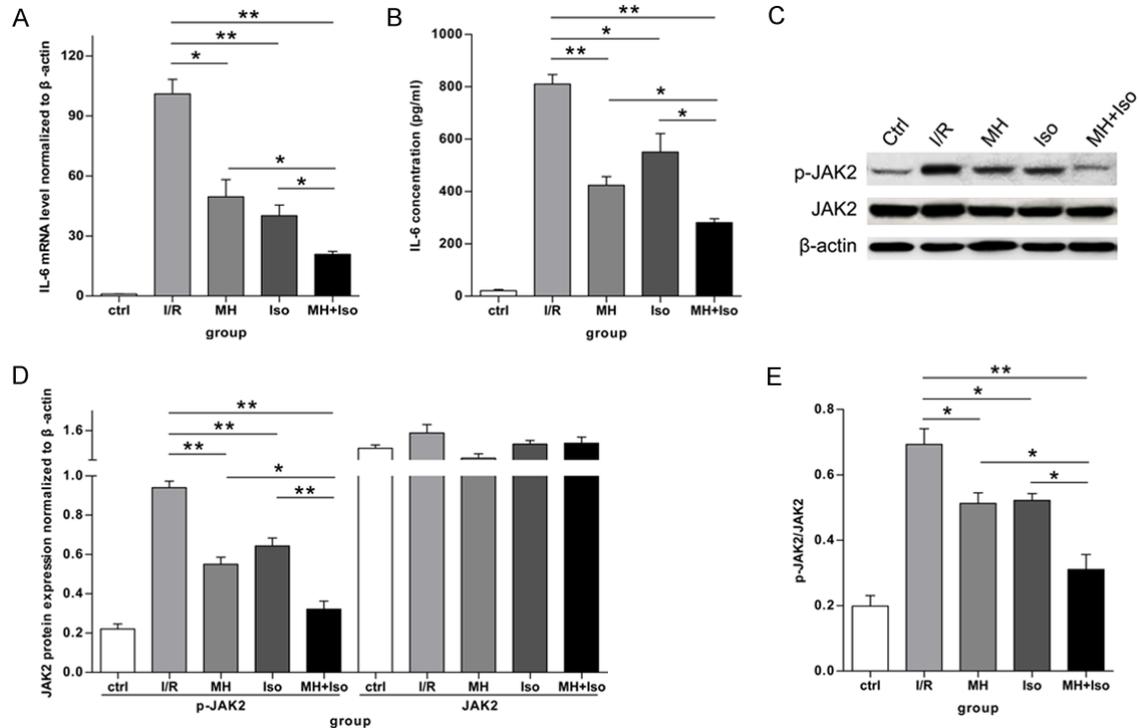


Figure 3. The mRNA level of IL-6 (A), protein levels of IL-6 (B), JAK2 and p-JAK2 (C, D) and JAK2 phosphorylation level (E), Data are expressed as mean \pm SD (n=5), *P < 0.05, **P < 0.01.

ELISA assay

The IL-6 level in the cerebral ischemic cortexes was determined by the IL-6 assay kit, according to the manufacturer's instructions.

Statistics

All data were expressed as mean \pm SD, and analyzed by SPSS 16.0. Comparisons among groups were analyzed by one-way ANOVA and comparison between two groups was conducted with SNK method. P < 0.05 or 0.01 was considered as statistically significant.

Results

Neurological deficit score and cerebral infarct volume

As reflected in **Figure 1**, there was a significant increase of the neurological deficit score (NDS) and cerebral infarct volume (CIV) in rats after 2 h MCAO induced ischemia and 24 h reperfusion. Compared with I/R group, the NDS and CIV in three post-treatment group (MH, Iso and MH+Iso group) were all significantly reduced (P

< 0.01). Treatment with MH+Iso was linked to more marked improvements in NDS and CIV than the treatment with MH or Iso alone (P < 0.01 or P < 0.05).

The expression of STAT3 and p-STAT3 in the cerebral ischemic cortexes

As can be seen from **Figure 2A-C**, the expression of STAT3 mRNA and protein in the cerebral ischemic cortexes in I/R group were significantly increased, and treatment with MH, Iso or MH+Iso could obviously reduce the expression (P < 0.05 or P < 0.01). As shown in **Figure 2D**, cerebral ischemia reperfusion caused a significantly increase in the level of STAT3 phosphorylation in cerebral cortex, which can be prevented by the treatment with MH, Iso or MH+Iso (P < 0.01), and the combined use of MH and Iso was more effective than one kind alone (P < 0.05 or P < 0.01).

The expression of IL-6, JAK2 and p-JAK2 in the cerebral ischemic cortexes

As shown in **Figure 3A, 3B**, expression of IL-6 mRNA and protein in cerebral cortex significant-

Cerebral ischemia-reperfusion injury protection

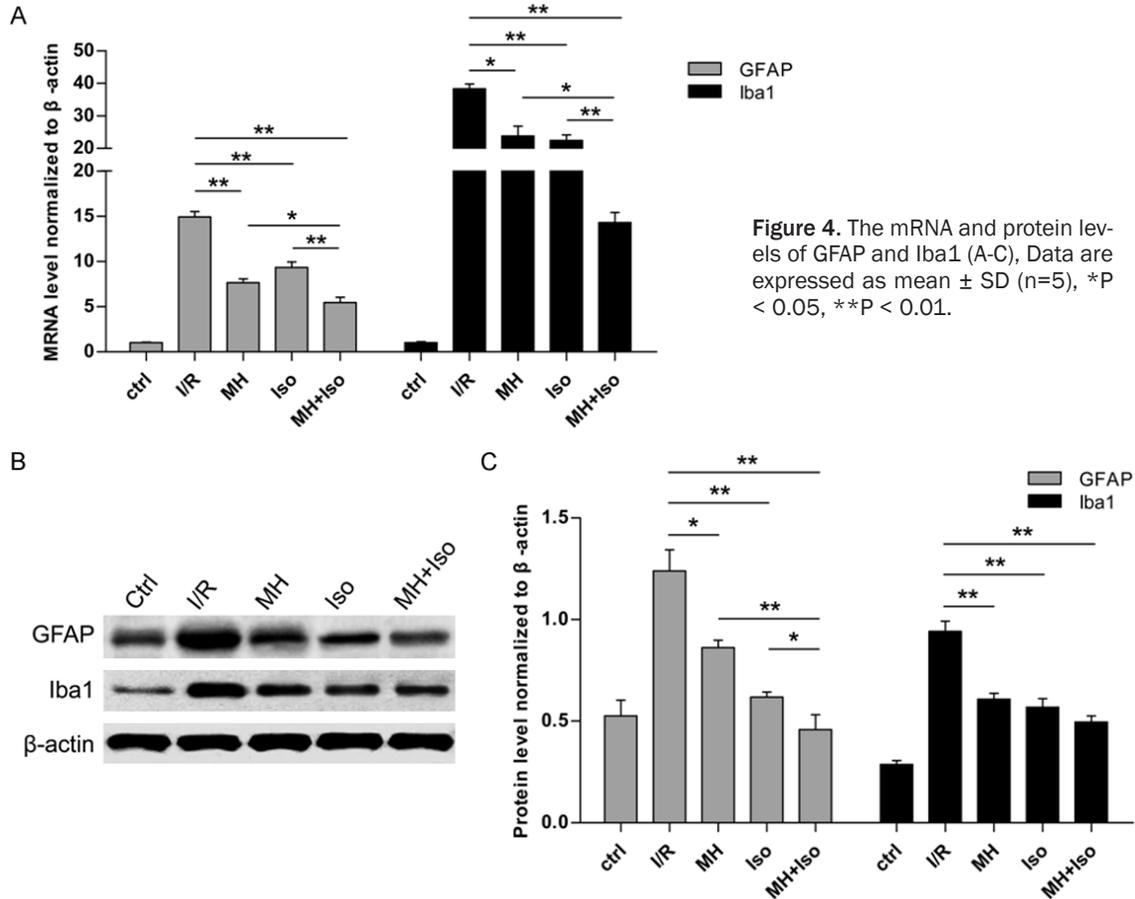


Figure 4. The mRNA and protein levels of GFAP and Iba1 (A-C), Data are expressed as mean \pm SD (n=5), *P < 0.05, **P < 0.01.

ly up-regulated when the rats were subjected to cerebral I/R. Treatment with MH, Iso or MH+Iso could significantly suppress IL-6 expression at the mRNA and protein level (P < 0.05 or P < 0.01). In addition, cerebral I/R caused an increased protein expression of p-JAK2 (P < 0.01), but had no effect on JAK2 protein expression, thus, leading to improvement of JAK2 phosphorylation level (Figure 3C-E). The increased phosphorylation level of JAK2 was reduced by all the three treatments (P < 0.01), among them, combination of MH and Iso was the most effective processing method (P < 0.05).

The expression of GFAP and Iba1 in the cerebral ischemic cortexes

The mRNA and protein expression of GFAP and Iba1 in the cerebral ischemic cortexes from I/R group were significantly increased, when compared with the control group (Figure 4). These indexes were apparently reduced in the three treatment groups (P < 0.05 or P < 0.01), and

approached to the minimum in the MH+Iso group.

Discussion

Cerebral ischemia/reperfusion (I/R) injury is a complex pathophysiological process, and the injury mechanisms associated are various, involves toxicity of excitatory amino acid [15], inflammatory reaction [16], oxidative stress [17], cell apoptosis [18], blood brain barrier damage [19], and leukocyte accumulation in blood vessels [20]. Especially, some researchers highlighted that cerebral I/R induced STAT3 activation plays a critical role in the brain tissue injury [4, 6]. In the present study, the expression of p-STAT3 mRNA and protein in the cerebral ischemic cortexes was significantly increased after 2 h ischemia and 24 h reperfusion, similar to the results of previous studies.

Interleukin-6 (IL-6) is pleiotropic cytokine, and has been implicated in various central nervous system disorders, such as stroke [21, 22]. Up

regulation expression of IL-6 in brain tissue after cerebral ischemia was also observed in some researches [23, 24]. Consistent with previous studies, expression of IL-6 mRNA and protein in cerebral cortex significantly up-regulated when the rats were subjected to cerebral I/R in this study. JAK-STAT is an important downstream signal pathway of IL-6. Binding of IL-6 to its receptors leads to dimerization of gp130, followed by phosphorylation of JAK2 and STAT3. Phosphorylated STAT3 (p-STAT3) dimerizes and translocates into nucleus, and binds to DNA to regulate target gene transcription. Our investigation showed that JAK2 phosphorylation level significantly increased at 24 h after ischemia, which corresponded to the temporal profile of IL-6 and STAT3 expression. So we speculated that excessive release of IL-6 active the JAK-STAT pathway in brain cell.

To study the cellular localization of JAK2 and STAT3 phosphorylation as a time of reperfusion following ischemia, numerous researches have been performed. However, the conclusion is inconsistent. Shigeaki Suzuki *et al.* found that phosphorylated STAT3 was first detected in neurons at 3.5 h after reperfusion and peaked at 24 h, and then in endothelial cells at 48 h of reperfusion [6]. Several other researches also observed STAT3 phosphorylation in neurons [5, 25]. Nevertheless, The research by Irawan Satriotomo *et al.* demonstrated that both p-JAK2 and p-STAT3 was basically localized in the macrophages/microglia after 6-72 h of reperfusion [4]. Jeong-Sun Choi *et al.* found STAT3 activation was in the reactive astrocytes 4 hours after ischemia [26]. This discrepancy may be due to the different experimental protocols and the JAK-STAT pathway regulatory system.

Glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1) are the specific marker of astrocyte and microglia, respectively. The results of this study showed that expression of GFAP and Iba1 were up-regulated significantly after cerebral I/R, indicating that the astrocyte and microglia maybe excessively activated after cerebral I/R. It has been reported that activation of the JAK2/STAT3 plays a key role in the induction of gliosis or glial proliferation in astrocytes and microglia [4, 27-30], and gliosis may contribute to the development of neuronal damage, although the conclusion is inconsistent [4, 31-33].

Therapeutic hypothermia is a common method for treating ischemic stroke and other types of brain injury [34, 35], and multiple genes and proteins related to cell growth, inflammation and apoptosis in the ischemic brain were involved in the underlying mechanism [36, 37]. The phosphorylation levels of JAK2 and STAT3 in mild hypothermia treatment groups were significantly decreased, when compared with the model group, suggesting mild hypothermia could effectively inhibit the activation of JAK/STAT molecular pathways, which was supported by the related literature [11]. Isoflurane is one of the most extensively used volatile anesthetics, and it has been repeatedly shown that isoflurane treatment induces ischemic tolerance in the brain [38-40]. However, the underlying mechanisms involved needs further investigation. In this work, isoflurane post processing significantly inhibited the phosphorylation of JAK2 and STAT3, indicating isoflurane could improve ischemic tolerance in brain tissue through restraining JAK/STAT pathway. Taken together, the underlying mechanism for protective effect of mild hypothermia and isoflurane post treatment against cerebral I/R injury may be that mild hypothermia and isoflurane post treatment inhibit the excessive activation of astrocyte and microglia after cerebral I/R through suppression of JAK2/STAT3 activation, eventually, protect the neurons from I/R injury.

In conclusion, the current study demonstrates that mild hypothermia combined with isoflurane post-treatment could alleviate cerebral I/R injury in rats through inhibition of STAT3 activation. Our results also reveal the crucial role of JAK2/STAT3 activation in cerebral I/R injury, and STAT3 maybe a potential target of cerebral I/R therapy.

Acknowledgements

This study was financially supported by the funds from Shanghai Health and Family Planning Commission (Grant No.: 20114218) and Science and Technology Committee of Minhang District, Shanghai (Grant No.: 2011MHZ45).

Disclosure of conflict of interest

None.

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