Enhanced oxidative stress response and neuroprotection of combined limb remote ischemic conditioning and atorvastatin after transient ischemic stroke in rats

Changhong Ren[1,2,3], Sijie Li[2], Kaiyin Liu[4], Gary B. Rajah[4], Anbo Zhang[2], Rongrong Han[1,2,3], Yuanyuan Liu[5], Qingjian Huang[1,2,3], Haiyan Li[1,2,3], Yuchuan Ding[4], Xunming Ji[2,3]

Abstract:

BACKGROUND: Limb remote ischemic conditioning (LRIC) and atorvastatin (AtS) both provide neuroprotection in stroke. We evaluated the enhanced neuroprotective effect of combining these two treatments in preventing ischemia/reperfusion (I/R)-induced cerebral injury in a rat model and investigated the corresponding molecular mechanisms.

MATERIALS AND METHODS: Transient cerebral ischemia was induced in Sprague–Dawley male rats by middle cerebral artery occlusion (MCAO) for 90 min followed by reperfusion (I/R). Rats were divided into 5 groups, sham, I/R, I/R + AtS, I/R + LRIC and I/R + AtS + LRIC. Pretreatment with LRIC and/or AtS for 14 days before MCAO surgery. Infarct volume, neurological score, Western blot, immuno-histochemical analyses were performed.

RESULTS: The combination of LRIC plus AtS pretreatment decreased infarct volume and inhibited neuronal apoptosis. Combination treatment achieved stronger neuroprotection than monotherapy with LRIC or AtS. These therapies reduced reactive oxygen species production in the peri-ischemia region, associated with significantly increased expression and activation of superoxide dismutase 1, hemeoxygenase 1 and nuclear factor erythroid 2-related factor 2.

CONCLUSIONS: Both LRIC and AtS + LRIC treatments conferred neuroprotection in ischemic stroke by reducing brain oxidative stress. AtS plus LRIC is an attractive translational research option due to its ease of use, tolerability, economical, and tremendous neuroprotective potential in stroke.

Keywords:

Atorvastatin, ischemia/reperfusion, limb remote ischemic conditioning, nuclear factor erythroid 2-related factor 2 pathway, oxidative stress

[1]Institute of Hypoxia Medicine, Xuanwu Hospital, Capital Medical University, Beijing, China, [2]Beijing Key Laboratory of Hypoxia Conditioning Translational Medicine, Beijing, China [3]Center of Stroke, Beijing Institute for Brain Disorder, Beijing, [5]Department of Endocrinology, Beijing, China, Huai'an First People's Hospital, Nanjing Medical University, Huai'an 223300, Jiangsu Province, China, [4]Department of Neurosurgery, Wayne State University School of Medicine, Detroit, MI 48201, USA

Address for correspondence:
Dr. Xunming Ji, Department of Neurosurgery, Xuanwu Hospital, Capital Medical University, Chang Chun Road 45, Beijing 100053, China.
E-mail: jixm@ccmu.edu.cn

Introduction

Ischemic stroke, which results from sudden interruption of the blood supply to areas of the brain, remains one of the leading causes of death and a significant cause of morbidity worldwide.[1] Endovascular thrombectomy studies have demonstrated that timely reperfusion remains the only effective treatment following cerebral ischemia.[1,2] Currently, most effective reperfusion options are limited to use of systemic thrombolysis with intravenous tissue-type plasminogen activator for smaller vessel disease and *in situ* clot retrieval for large vessel occlusion approved by the Food and Drug Administration.[3] However, reperfusion with oxygenated blood following ischemia also potentiates ischemic damage.[4] Specifically, reactive oxygen species (ROS) formed during the early phase of reperfusion,

augmenting neuronal injury.[5] Thus, there is a strong impetus to discover with novel approaches for protecting the brain from ischemia/reperfusion (I/R) damage.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a regulator of the antioxidant cell defense system.[6] Nrf2 binds to antioxidant response element (ARE) and activates the transcription of antioxidant stress genes, which contributes to cytoprotection in oxidative stress-induced injury with cerebral I/R.[7] Previous evidence showed that Nrf2 knockout (Nrf2$^{-/-}$) mice are more vulnerable to the cytotoxic effects of oxidative stress-induced brain injury compared with wild-type mice.[8] Cu-Zn superoxide dismutase (SOD1), an effective anti-oxidant enzyme, is one of the downstream effector enzymes of the Nrf2 pathway.[9] SOD1 catalyzes the dismutation of superoxide anions to hydrogen peroxide. Recent studies have shown significant therapeutic effects of nanoformulated SOD1 on preclinical models of reperfusion injury after ischemic stroke.[10] Hemeoxygenase 1 (HO-1) is another downstream effector enzyme of the Nrf2 pathway, which has been reported to exert an antioxidant effect and thereby prevents against apoptosis.[11] Thus, clinical interventions could be targeted toward controlling and modulating these cellular responses against oxidative stress.

Several studies have demonstrated that limb remote ischemic conditioning (LRIC) decreases I/R-induced injury.[12-14] Our recent proof of concept randomized clinical study showed that LRIC treatment twice daily for 2 weeks before carotid artery stenting (CAS) could decrease ischemic brain injury.[15] Thus, LRIC, as a simple, convenient, and inexpensive physical therapy modality, has potential clinical neuroprotective role in ameliorating reperfusion injury. Statins, as lipid synthesis regulators, also exerts well documented benefits on ischemic stroke since their introduction into clinical therapy in the late 1980s.[16] Accumulating evidence indicates that atorvastatin (AtS) can exert neuroprotection by modifying anti-oxidative pathway and inflammatory responses after stroke.[17,18] Moreover, the use of AtS has widely been explored as pretreatment in stroke prevention after cerebrovascular accidents.[19] Taken together, given the antioxidative and neuroprotective effects of both AtS and LRIC during I/R-induced injury after stroke, we aimed at determining the therapeutic effects of monotherapy with LRIC or AtS and combination treatment of AtS + LRIC in stroke prevention by modulating oxidative stress through regulatory mediators of Nrf2 pathway.

Materials and Methods

Animal
All animal experiments were approved by Animal Care and Use Committee of Xuanwu Hospital, Capital Medical University, China, and conducted according to the National Institutes of Health guidelines. Fifty adult male Sprague–Dawley rats (180–200 g weight) were purchased from Vital River Laboratories, Beijing, China, and maintained on a 12-h light/dark cycle with unlimited access to food and water. The animals were randomly divided the following groups: AtS-treatment group (AtS group); LRIC treatment group (LRIC group); AtS and LRIC combined treatment group (AtS + LRIC group); Vehicle group, where 0.5% methyl cellulose was administered. Middle cerebral artery occlusion (MCAO) surgery [Figure 1] was performed 14 days later.

Atorvastatin administration
AtS was suspended in 0.5% methyl cellulose, and 20 mg/kg was administered through oral gastric tube every day up to 14 days as previously described.[19]

Limb remote ischemic conditioning
LRIC were accomplished as our previously described. In brief, A tourniquet (8 mm) was tightened around the upper thigh for 3 cycles, with each occlusion or release phase lasting 10 min.[20] Rats were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneally) before LRIC treatment, as previously described.[21] LRIC was applied every day up to 14 days before MCAO. The sham and ischemic control groups received the same dosage of sodium pentobarbital.

Focal cerebral ischemia and reperfusion
Rats were anesthetized and MCAO was induced by intraluminal occlusion for 90 min using a Nylon monofilament suture as previously described.[22] In brief, the right common carotid artery and the right external carotid artery (ECA) were exposed. The ECA was then dissected distally, ligated, and coagulated. The middle cerebral artery (MCA) was occluded using a heparinized intraluminal filament (0.28 mm, rounded tip). After 90 min, the suture was withdrawn. During the operation, rectal temperature was maintained at 37°C ± 0.5°C with a thermostat-controlled heating blanket. Sham-operated mice underwent the identical surgery except that the MCA was not occluded.

Infarct size measurement

Infarct size was measured according to previous methods.[14] Twenty-four hours after surgery, the rats were anesthetized with 1% chloral hydrate, and then, the brains were removed and sectioned coronally at the level of optic chiasm at 2 mm intervals, generating a total of six sections, which were stained with 2% solution of 2, 3, 4-triphenytetrazolium-chloride (TTC). Using a computerized image analysis system (Image-Pro Plus, version 5.1, Media Lybernetics, Silver Spring, MD, USA), the area of infarction was defined at the sides of
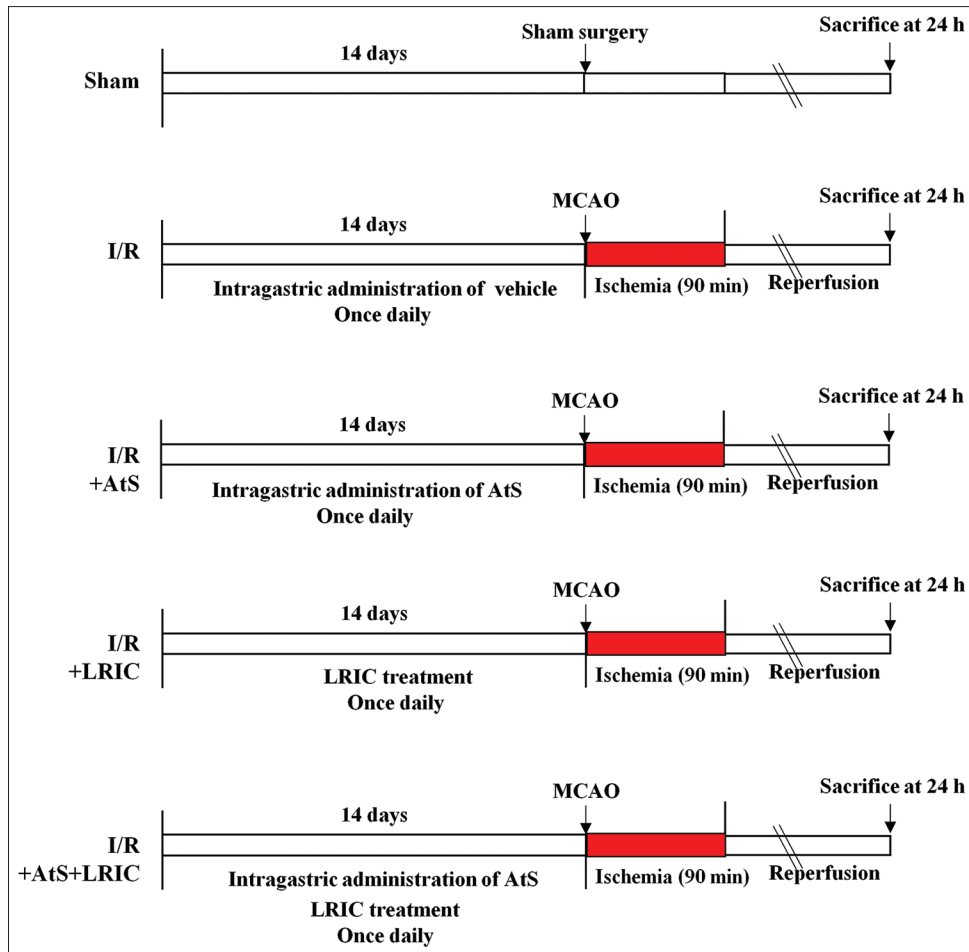
Figure 1: The representative sketches of experiment group. AtS: Atorvastatin; LRIC: limb remote ischemic conditioning; I/R: ischemia/reperfusion

the inner section. Infarct size of the ischemic region was normalized to the nonischemic region and expressed as a percentage, and an average value from the six slices was presented.

Neurobehavioral test

Neurological deficit was determined using the neurobehavioral scoring system developed by Belayev *et al.*[23] with modification. The scoring system was graded on a scale of 0–12 (minimal score, 0; maximal score, 12). The tests included (1) postural reflex test, to examine upper body posture, and (2) the forelimb placing test, to examine sensorimotor integration. Ten rats were used for each group.

The elevated body swing test (EBST) was used to test asymmetric motor behavior.[24] The rats were held at the base of the tail and raised 15 cm above the testing surface. The initial direction of swing is defined as the turning of the upper body by >10 degrees to either side, and was recorded in 30 trials/rat. The number of turns to each direction (left or right) was recorded for each rat. The total number of swings made to the left was divided by

30 (*n* number of trials) to get a percentage of left-biased swings. Ten rats were used for each group.

The observer was blinded to the experimental conditions while performing the neurobehavioral tests.

Terminal deoxynucleotidyl transferase mediated nick end labeling assay

Each group, rats (*n* = 5 per group) were anesthetized and decapitated at 24 h after reperfusion. Neural apoptosis was assessed in 10-µm frozen coronal sections using the *in situ* Cell Death Detection Kit-POD (Roche, San Francisco, CA) following the manufacturer's instructions. Green-staining cells in the peri-infarct region were counted as apoptotic cells. The quantitative analysis was expressed using the average of three brain slides, and each slide included four random fields.

Measurement of intracellular reactive oxygen species

The fluorescent probe dihydroethidium (DHE) (Vigorous Biotechnology Beijing Co., Ltd, Beijing, China) was used to measure ROS production as described

previously[25] with modifications. Frozen coronal brain sections (10 μm) were incubated for 2 h in 5% bovine serum albumin, and subsequently incubated in 100 μmol/L DHE for 60 min in the dark. After washing three times with phosphate buffered saline, sections were counterstained with 4',6-diamidino-2-phenylindole to visualize nuclei and then examined by fluorescence microscopy (Nikon, Japan).

Western blot

Protein was isolated from the rat peri-infarct region at 24 h after reperfusion. Protein (50 μg) was electrophoresed on 12.5% sodium dodecyl sulfate polyacrylamide gels, and then transferred to a polyvinylidene difluoride membrane (Millipore Corporation, USA). Membranes were blocked for 1 h in 5% skim milk in Tris-buffered saline with Tween-20 buffer and immersed overnight at 4°C with primary antibodies against HO-1 (1:1000; Santa Cruz), SOD1 (1:500; abcam), Nrf-2 (1:500; abcam)), respectively. β–actin was used to verify equal protein loading. The specific reaction was visualized by the chemiluminescence substrate luminol reagent (GE Healthcare, UK). The optical density of protein was measured using Image-Pro Plus software 5.0 (Rockville, MD, USA) according to the manufacturer's instructions. The mean amount of protein expression from the sham group was arbitrarily assigned a value of 1 to serve as reference.

Measurement of superoxide dismutase

Periinfarct region homogenates at 24 h after reperfusion were used for SOD activity measurement. SOD1 activity was assayed by using the SOD1 Assay Kit-WST (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturers' protocols.

Statistical analysis

Data were expressed as mean and standard error (SE) (mean ± SE) and statistical tests were performed with SPSS for Windows, version 19.0 (SPSS Inc. IBM). For comparison between two groups, Student's *t*-test was used. The differences among groups were assessed using one-way ANOVA with a significance level at $P < 0.05$. *Post hoc* comparison among groups was further performed using the least significant difference method.

Results

To explore whether LRIC and AtS pretreatment prevent I/R-induced injury, infarct size was measured at 24 h after reperfusion. Infarct volume was significantly reduced in the AtS and LRIC monotherapy group compared with I/R control group ($P < 0.05$) [Figure 2a]. AtS + LRIC combination further reduced brain injury ($P < 0.01$) [Figure 2a]. In addition, AtS + LRIC combination significantly reduced infarct volume as compared with AtS or LRIC alone ($P < 0.05$) [Figure 2a].

To further analyze the neuroprotective effect of AtS and LRIC pretreatment, DNA fragmentation in brain tissues after ischemia was performed using terminal deoxynucleotidyl transferase mediated nick end labeling (TUNEL) assay. The number of TUNEL-positive cells in the peri-infarct region in the I/R control group was significantly increased compared to the sham group ($P < 0.01$) [Figure 2b]. Although only LRIC monotherapy slightly attenuated TUNEL-positive cells, AtS + LRIC treatment significantly decreased the number of TUNEL-positive cells compared with the control group ($P < 0.01$) [Figure 2b]. There was no significant difference between LRIC alone and the AtS + LRIC groups ($P > 0.05$).

Next, we asked whether combining AtS and LRIC treatment would affect the neurological functional outcome. EBST showed that AtS + LRIC treatment improved asymmetric motor behavior at 24 h after reperfusion [Figure 3a]. However, neither LRIC nor AtS alone was sufficient to reduce neurological deficits [Figure 3a]. As shown in Figure 3b, neurological deficits, including body posture and sensorimotor integration, were significantly improved in the AtS + LRIC group, compared with I/R control group [Figure 3b]. Similarly, neither LRIC nor AtS alone was sufficient to reduce neurological deficits [Figure 3b].

To determine whether AtS + LRIC treatment can attenuate oxidative stress-induced by cerebral I/R, ROS production was evaluated. DHE staining showed that I/R significantly increased ROS production compared with sham group at 24 h after reperfusion ($P < 0.01$) [Figure 4]. Both AtS and LRIC alone were able to significantly decrease ROS production ($P < 0.05$). Combination treatment further induced a large ($P < 0.01$) reduction in ROS levels, suggesting an attenuated oxidative damage.

Then, we explored the mechanism underlying combination therapy-mediated neuroprotection after ischemic stroke. We first analyzed the SOD1 activity at 24 h after reperfusion by biochemical analyses. AtS + LRIC combination significantly increased SOD1 expression compared with I/R group ($P < 0.01$) [Figure 5a]. By Western blot analysis, we found that AtS and LRIC alone slightly upregulated SOD1 expression ($P < 0.05$). AtS + LRIC combination significantly increased SOD1 expression ($P < 0.01$) [Figure 5b]. Furthermore, AtS + LRIC combination significantly upregulated SOD1 expression as compared with AtS or LRIC alone ($P < 0.01$). Then, we assessed HO-1 protein level. AtS alone was not able to significantly increase the HO-1 expression. LRIC alone was only able to slightly increased HO-1 expression ($P < 0.05$). AtS + LRIC combination significantly raised HO-1 expression ($P < 0.05$). AtS + LRIC combination also significantly upregulated HO-1 expression as compared
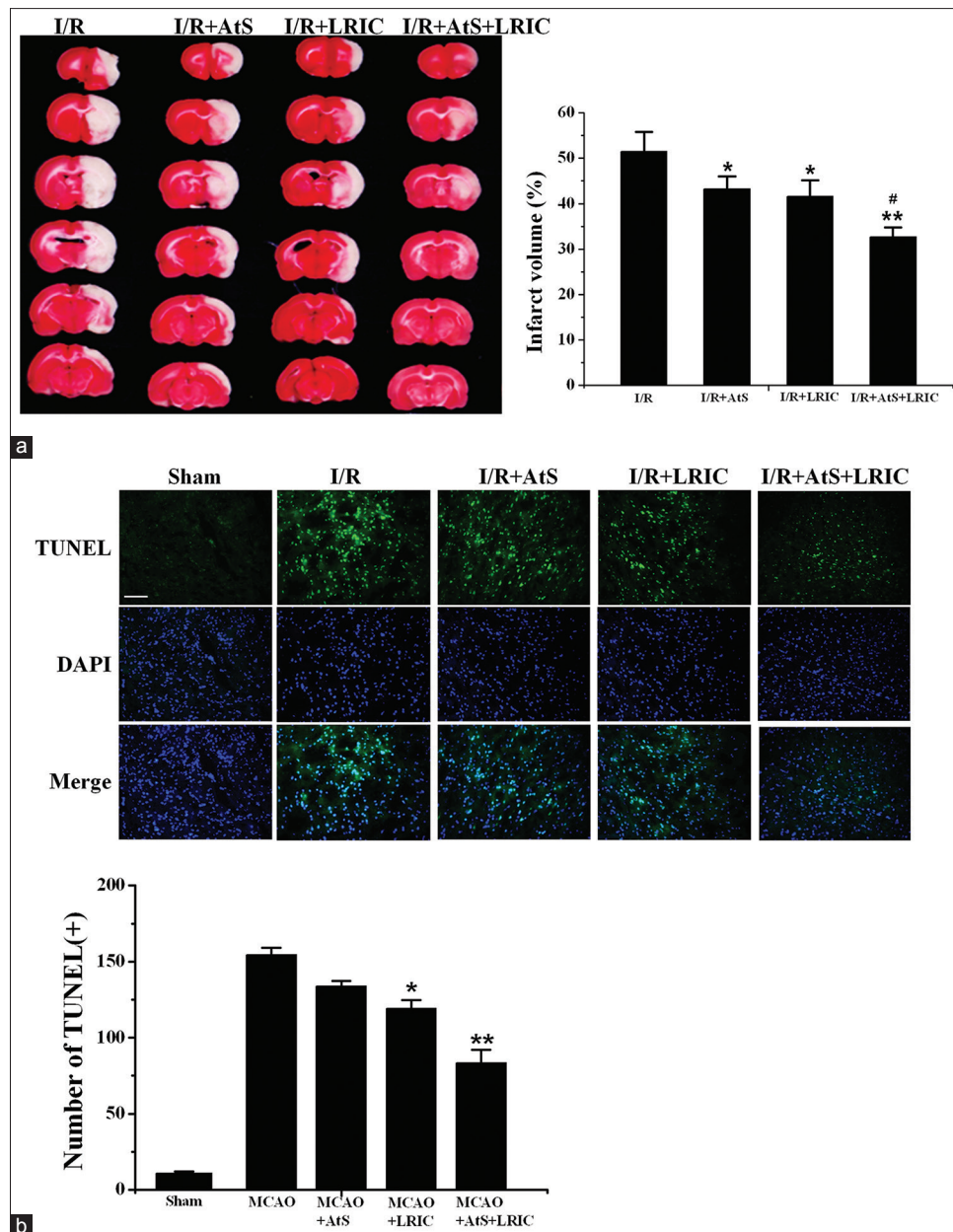
Figure 2: Atorvastatin and limb remote ischemic conditioning combination protects against ischemia/reperfusion injury in rat. (a) Cerebral infarct volume evaluated by 2, 3, 5-triphenyltetrazolium chloride staining of coronal brain sections ($n = 8$). *$P < 0.05$, **$P < 0.01$, versus ischemia/reperfusion group. #$P < 0.05$, versus ischemia/reperfusion + limb remote ischemic conditioning group. (b) Neuronal apoptosis in the peri-infarct region detected by terminal deoxynucleotidyl transferase mediated nick end labeling and 4',6-diamidino-2-phenylindole double staining ($n = 4$). * $P < 0.05$, ** $P < 0.01$, versus middle cerebral artery occlusion group. Scale bar = 100 μm

with AtS or LRIC alone ($P < 0.05$) [Figure 5c]. In addition, the monotherapy with AtS or LRIC only mildly increased Nrf2 levels [Figure 5d]. As above, the combined treatment was successful in increasing the Nrf2 levels ($P < 0.01$). Compared with I/R + LRIC group, AtS + LRIC combination significantly increased Nrf2 levels ($P < 0.01$) [Figure d].

Discussion

In this study, we first discovered that a combination of LRIC and AtS pretreatment ameliorated the cerebral I/R

injury and oxidative stress. In addition, combination of LRIC and AtS pretreatment increased the expression of SOD1, HO-1 and Nrf2 at day 1 following I/R. These results suggest that combination therapy has beneficial effects on anti-oxidative stress mediators in AtS + LRIC mediated neuroprotection after I/R.

Restoration of cerebral blood flow is the only effective therapeutic strategy after ischemic stroke, however, prevention of reperfusion injury still remains a daunting challenge for ischemic stroke therapy.[5] The recent report showed that LRIC treatment twice
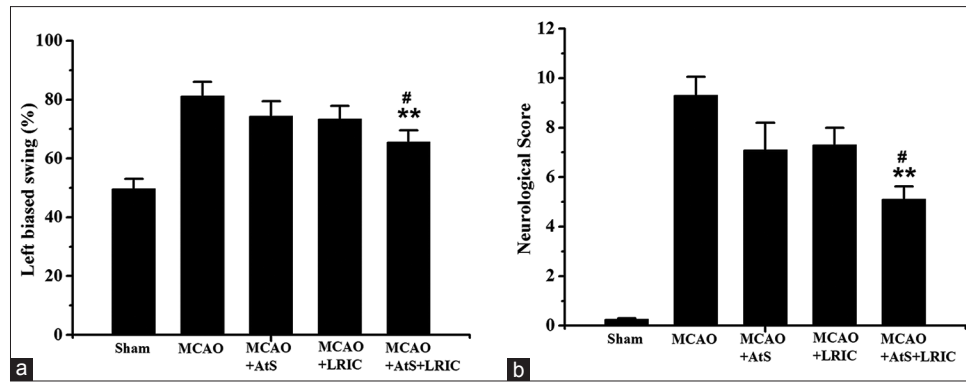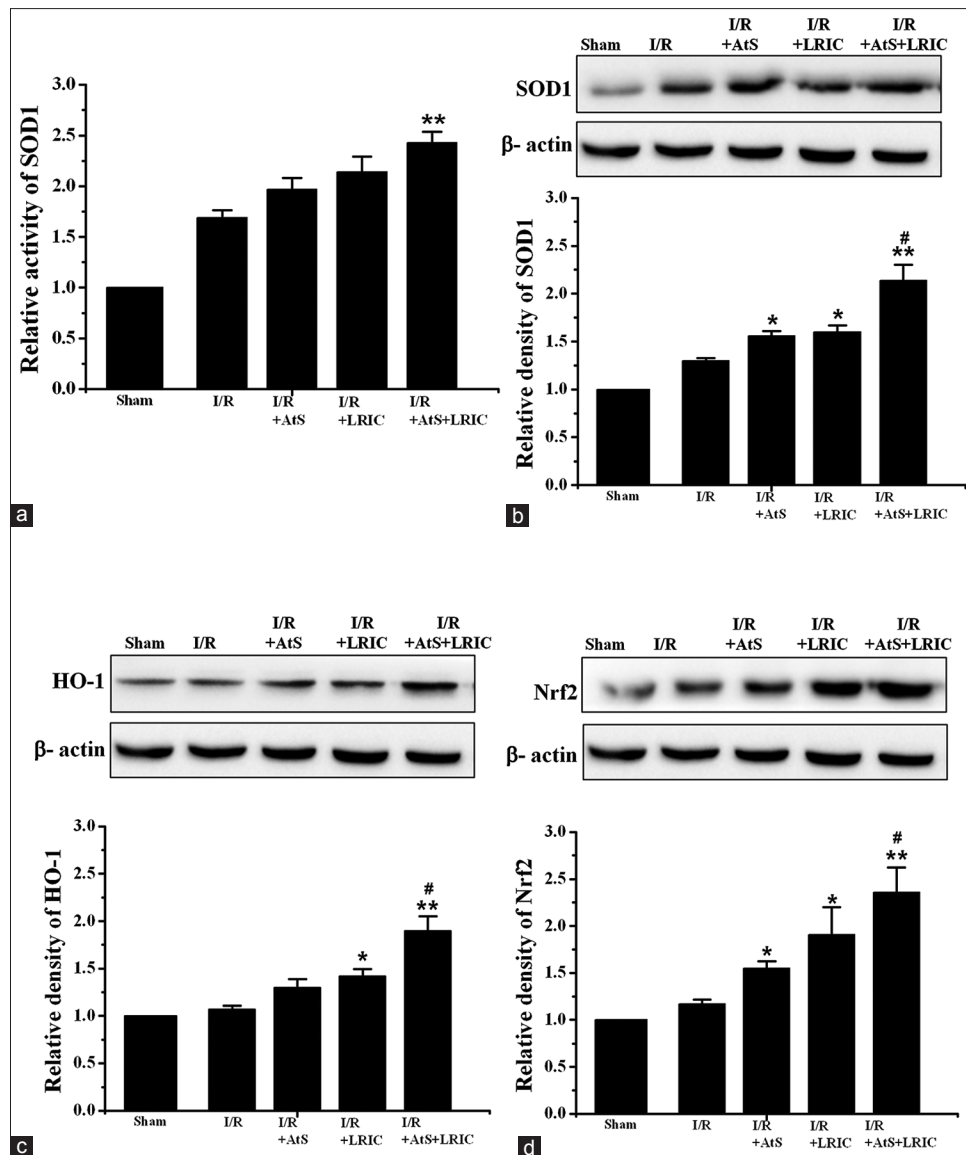
Figure 3: Atorvastatin and limb remote ischemic conditioning combination attenuated neurological deficiency. (a) Elevated body swing test (higher percentage correspond to more severe deficits) (*n* = 8). **$P < 0.01$, versus ischemia/reperfusion group. #$P < 0.05$, versus ischemia/reperfusion + limb remote ischemic conditioning group. (b) Neurological deficits were determined using neurobehavioral scoring system (higher scores correspond with more severe deficits) (*n* = 8). **$P < 0.01$, versus ischemia/reperfusion group. #$P < 0.05$, versus ischemia/reperfusion + limb remote ischemic conditioning group
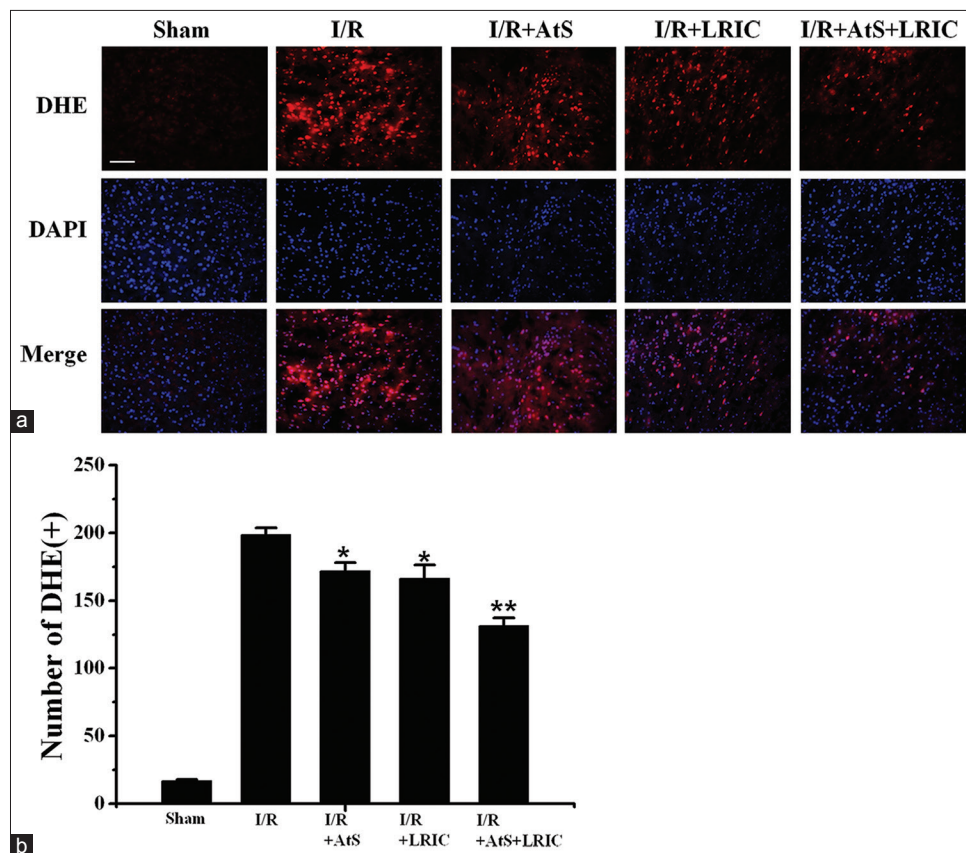
Figure 4: Atorvastatin and limb remote ischemic conditioning combination oxidative stress after ischemia/reperfusion in mice. (a) Reactive oxygen species level in the peri-infarct region was detected by dihydroethidium and 4',6-diamidino-2-phenylindole double staining (*n* = 5). (b) Relative reactive oxygen species levels in each group. *$P < 0.05$, **$P < 0.01$, versus ischemia/reperfusion group. #$P < 0.05$, versus ischemia/reperfusion + limb remote ischemic conditioning group. Scale bar = 100 μm

daily for 2 weeks before CAS was able to decrease ischemic brain injury secondary to CAS.[15] Although the underlying mechanism is still unclear, this therapeutic approach suggested the beneficial effect of LRIC in preventing cerebral ischemic injury. In this study, we found that LRIC pretreatment for 2 weeks before focal cerebral ischemia in rats has the similar neuroprotection. ROS leads to harmful effects through

direct damage of cellular proteins, lipids, and DNA in the brain during and after I/R.[26] Our further analysis showed that pretreatment with LRIC before transient ischemia reduced ROS generation. ROS was reported to induce apoptosis during reperfusion.[5] Apoptosis, which has been frequently observed in animal models of I/R-induced cerebral injury, is crucial characteristic of I/R-induced tissue injury.[5] The present study

Figure 5: Atorvastatin and limb remote ischemic conditioning combination upregulates antioxidant expression and activity in the peri-ischemic region after ischemia/reperfusion injury. (a) Effect of atorvastatin and limb remote ischemic conditioning on superoxide dismutase activity ($n = 4$), as detected by biochemical methods ($n = 4$). **$P < 0.01$, versus ischemia/reperfusion group. (b) superoxide dismutase protein level in peri-infarct region, as determined by Western blotting ($n = 4$). *$P < 0.05$, **$P < 0.01$, versus ischemia/reperfusion group. #$P < 0.05$, versus ischemia/reperfusion + atorvastatin, or ischemia/reperfusion + limb remote ischemic conditioning group. (c) Hemeoxygenase 1 protein level in peri-infarct region, as determined by Western blotting ($n = 4$). *$P < 0.05$, **$P < 0.01$, versus ischemia/reperfusion group. #$P < 0.05$, versus ischemia/reperfusion + atorvastatin, or ischemia/reperfusion + limb remote ischemic conditioning group. (d) Nuclear factor erythroid 2-related factor 2 protein level in peri-infarct region, as determined by Western blotting ($n = 4$). *$P < 0.05$, **$P < 0.01$, versus ischemia/reperfusion group. #$P < 0.05$, versus ischemia/reperfusion + atorvastatin group

evaluated the number of apoptosis cells in peri-ischemia region. LRIC pretreatment significantly decreased TUNEL-positive cells compared with I/R group, which suggested that LRIC reduced apoptosis. In addition, TTC staining of brain slices at 24 h after reperfusion showed significant reduction in infarct size for the combination treatment as compared to I/R group suggesting that both apoptosis and necrosis pathways were reduced. Although the measures here cannot definitively explain the better outcomes, our findings support a role of LRIC in reducing oxidative stress-induced cell death.

Statins are used clinically primarily for their lipid lowering properties related to HMG CoA reductase inhibition, which is well used in the prevention of recurrent stroke.[27] In addition to their their lipid lowering properties, statin shows various pleiotropic effects such as anti-inflammatory, anti-oxidative, and atherosclerosis preventing effects.[28] AtS has antioxidant properties such as scavenging ROS,[29] inhibiting ROS-induced DNA fragmentation, and restraining superoxide generation in the blood vessel.[18] Based on the role of AtS in preventing recurrent stroke and reducing stress injury, we attempted to explore

whether the combined application of LRIC and AtS had synergistic effect in preventing ischemic reperfusion injury through reducing oxidative stress. In this study, we demonstrated that combination therapy using LRIC plus AtS produced enhanced neuroprotection compared with either treatment alone. This was evident both molecularly showing significant decrease in ROS generation and enhanced SOD1, Nrf2/HO-1 expression as well as functionally showing reduced infarct volume and better neurological outcomes after cerebral ischemia.

Blood flow reperfusion results in excess production of ROS, including superoxide radicals and peroxides. These ROS products, in turn, play a significant role in brain I/R injury.[5] The beneficial effect of combination therapy on reduced oxidative stress, in our study, may be attributed to its effect on SOD1, HO-1 and Nrf2, which are important enzymes related to ROS production and oxidative metabolism. As the most crucial endogenous antioxidant enzyme, SOD 1 scavenges oxygen free radicals and prevents DNA damage, initiation of the apoptotic pathway and cytotoxic mitochondrial damage. The improved activities of these endogenous antioxidant enzymes provide protection against oxidative stress. The present study demonstrates that although LRIC and AtS monotherapies both seem helpful in reducing oxidative stress in I/R injury, their combination produces further increase in SOD1 activity and protein expression. Nrf2 has been reported to be a key regulator in cell survival mechanisms.[30] Shah *et al*. reported that the Nrf2/HO-1 signaling pathway was tightly associated with ROS scavenging during the process of oxidative stress.[31] This study's findings show that LRIC monotherapy increased the Nrf2/HO-1 expression. This observation is consistent with previous findings that daily LRIC administration increases Nrf2/HO-1 expression after retinal ischemia.[32] The combination therapy of LRIC and AtS further increased the expression of Nrf2/HO-1. Integration of our results with previous findings and literature suggests that LRIC or AtS + LRIC exerts its effects though regulation of endogenous antioxidant system.

Conclusions

Combination therapy of LRIC and AtS is a powerful neuroprotective option in ischemia-reperfusion injury that is inexpensive, simple to use, and widely available. As such, combination therapy has high translational potential and is a promising therapeutic target for reducing neuronal damage after I/R injury. However, further studies are still required to better understand this novel treatment. Additional mechanistic studies will be needed to help clarify the neuroprotection demonstrated by combinative therapy of LRIC and AtS.

Conflicts of interest

There are no conflicts of interest.

References

1. Writing Group Members, Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, *et al.* Executive summary: Heart disease and stroke statistics–2016 update: A Report from the American Heart Association. Circulation 2016;133:447-54.
2. Savitz SI, Baron JC, Yenari MA, Sanossian N, Fisher M. Reconsidering neuroprotection in the reperfusion era. Stroke 2017;48:3413-9.
3. Moskowitz MA, Lo EH, Iadecola C. The science of stroke: Mechanisms in search of treatments. Neuron 2010;67:181-98.
4. Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. J Pathol 2000;190:255-66.
5. Jung JE, Kim GS, Chen H, Maier CM, Narasimhan P, Song YS, *et al.* Reperfusion and neurovascular dysfunction in stroke: From basic mechanisms to potential strategies for neuroprotection. Mol Neurobiol 2010;41:172-9.
6. Shelton P, Jaiswal AK. The transcription factor NF-E2-related factor 2(Nrf2): A protooncogene? FASEB J 2013;27:414-23.
7. Nguyen T, Nioi P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J Biol Chem 2009;284:13291-5.
8. Shah ZA, Li RC, Ahmad AS, Kensler TW, Yamamoto M, Biswal S, *et al*. The flavanol (-)-epicatechin prevents stroke damage through the Nrf2/HO1 pathway. J Cereb Blood Flow Metab 2010;30:1951-61.
9. Dreger H, Westphal K, Weller A, Baumann G, Stangl V, Meiners S, *et al.* Nrf2-dependent upregulation of antioxidative enzymes: A novel pathway for proteasome inhibitor-mediated cardioprotection. Cardiovasc Res 2009;83:354-61.
10. Jiang Y, Brynskikh AM, S-Manickam D, Kabanov AV. SOD1 nanozyme salvages ischemic brain by locally protecting cerebral vasculature. J Control Release 2015;213:36-44.
11. Kundu J, Kim DH, Kundu JK, Chun KS. Thymoquinone induces heme oxygenase-1 expression in HaCaT cells via Nrf2/ARE activation: Akt and AMPKα as upstream targets. Food Chem Toxicol 2014;65:18-26.
12. Gidday JM. Cerebrovascular ischemic protection by pre- and post-conditioning. Brain Circ 2015;1:97-103.
13. Ren C, Li N, Wang B, Yang Y, Gao J, Li S, *et al.* Limb ischemic perconditioning attenuates blood-brain barrier disruption by inhibiting activity of MMP-9 and occludin degradation after focal cerebral ischemia. Aging Dis 2015;6:406-17.
14. Ren C, Liu K, Cui X, Gao J, Ji X, Ding Y. Neural transmission pathways are involved in the neuroprotection induced by post but not per-ischemic limb remote conditioning. Brain Circ 2016;1:160-7.
15. Zhao W, Meng R, Ma C, Hou B, Jiao L, Zhu F, *et al.* Safety and efficacy of remote ischemic preconditioning in patients with severe carotid artery stenosis before carotid artery stenting: A Proof-of-concept, randomized controlled trial. Circulation

2017;135:1325-35.

16. Grundy SM. HMG-coA reductase inhibitors for treatment of hypercholesterolemia. N Engl J Med 1988;319:24-33.

17. Hong H, Zeng JS, Kreulen DL, Kaufman DI, Chen AF. Atorvastatin protects against cerebral infarction via inhibition of NADPH oxidase-derived superoxide in ischemic stroke. Am J Physiol Heart Circ Physiol 2006;291:H2210-5.

18. García-Bonilla L, Campos M, Giralt D, Salat D, Chacón P, Hernández-Guillamon M, *et al.* Evidence for the efficacy of statins in animal stroke models: A meta-analysis. J Neurochem 2012;122:233-43.

19. Hayashi T, Hamakawa K, Nagotani S, Jin G, Li F, Deguchi K, *et al.* HMG coA reductase inhibitors reduce ischemic brain injury of wistar rats through decreasing oxidative stress on neurons. Brain Res 2005;1037:52-8.

20. Ren C, Wang P, Wang B, Li N, Li W, Zhang C, *et al.* Limb remote ischemic per-conditioning in combination with post-conditioning reduces brain damage and promotes neuroglobin expression in the rat brain after ischemic stroke. Restor Neurol Neurosci 2015;33:369-79.

21. Ren C, Li S, Wang B, Han R, Li N, Gao J, *et al.* Limb remote ischemic conditioning increases notch signaling activity and promotes arteriogenesis in the ischemic rat brain. Behav Brain Res 2016. pii: S0166-4328(16) 30901-9.

22. Zwagerman N, Plumlee C, Guthikonda M, Ding Y. Toll-like receptor-4 and cytokine cascade in stroke after exercise. Neurol Res 2010;32:123-6.

23. Belayev L, Alonso OF, Busto R, Zhao W, Ginsberg MD. Middle cerebral artery occlusion in the rat by intraluminal suture. Neurological and pathological evaluation of an improved model. Stroke 1996;27:1616-22.

24. Borlongan CV, Sanberg PR. Elevated body swing test: A new behavioral parameter for rats with 6-hydroxydopamine-induced hemiparkinsonism. J Neurosci 1995;15:5372-8.

25. Liu P, Zhao H, Wang R, Wang P, Tao Z, Gao L, *et al.* MicroRNA-424 protects against focal cerebral ischemia and reperfusion injury in mice by suppressing oxidative stress. Stroke 2015;46:513-9.

26. Schaller B, Graf R. Cerebral ischemia and reperfusion: The pathophysiologic concept as a basis for clinical therapy. J Cereb Blood Flow Metab 2004;24:351-71.

27. Amarenco P, Labreuche J. Lipid management in the prevention of stroke: Review and updated meta-analysis of statins for stroke prevention. Lancet Neurol 2009;8:453-63.

28. George SJ, Dhond AJ, Alderson SM, Ezekowitz MD. Neuroprotective effects of statins may not be related to total and low-density lipoprotein cholesterol lowering. Am J Cardiol 2002;90:1237-9.

29. Kishi T, Hirooka Y, Shimokawa H, Takeshita A, Sunagawa K. Atorvastatin reduces oxidative stress in the rostral ventrolateral medulla of stroke-prone spontaneously hypertensive rats. Clin Exp Hypertens 2008;30:3-11.

30. Owuor ED, Kong AN. Antioxidants and oxidants regulated signal transduction pathways. Biochem Pharmacol 2002;64:765-70.

31. Shah ZA, Li RC, Thimmulappa RK, Kensler TW, Yamamoto M, Biswal S, *et al.* Role of reactive oxygen species in modulation of Nrf2 following ischemic reperfusion injury. Neuroscience 2007;147:53-9.

32. Zhang X, Jizhang Y, Xu X, Kwiecien TD, Li N, Zhang Y, *et al.* Protective effects of remote ischemic conditioning against ischemia/reperfusion-induced retinal injury in rats. Vis Neurosci 2014;31:245-52.