Atorvastatin protects against cerebral ischemia/reperfusion injury through anti-inflammatory and antioxidant effects

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Abstract
In addition to its lipid-lowering effect, atorvastatin exerts anti-inflammatory and antioxidant effects as well. In this study, we hypothesized that atorvastatin could protect against cerebral ischemia/reperfusion injury. The middle cerebral artery ischemia/reperfusion model was established, and atorvastatin, 6.5 mg/kg, was administered by gavage. We found that, after cerebral ischemia/reperfusion injury, levels of the inflammation-related factors E-selectin and myeloperoxidase were upregulated, the oxidative stress-related marker malondialdehyde was increased, and superoxide dismutase activity was decreased in the ischemic cerebral cortex. Atorvastatin pretreatment significantly inhibited these changes. Our findings indicate that atorvastatin protects against cerebral ischemia/reperfusion injury through anti-inflammatory and antioxidant effects.

Key Words: nerve regeneration; brain injury; cerebral ischemia/reperfusion; atorvastatin; E-selectin; myeloperoxidase; superoxide dismutase; malondialdehyde; inflammation; free radicals; blood-brain barrier; stroke; NSFC grant; neural regeneration

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Introduction
Hyperlipidemia is an independent risk factor for cardio-cerebrovascular diseases, and atorvastatin is often used to lower lipid levels. In this study, we sought to examine whether statins can protect against cardio-cerebrovascular diseases in subjects with normal blood lipid levels. Stroke is a major threat to health, and is associated with high morbidity, disability and mortality[1]. There are a series of reactions following cerebral ischemia/reperfusion, such as inflammation and an increase in free radicals, which may trigger secondary injury in ischemic tissue[2-3]. Indeed, the inhibition of inflammation reduces tissue damage in ischemia[4-6]. Thus, understanding the roles of inflammation and free radicals in ischemia/reperfusion injury is therefore of great importance.

3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, known as statins, are widely used to reduce levels of low density lipoprotein-cholesterol. As lipid-lowering drugs, statins exert neuroprotective effects on ischemic stroke. Exploring the mechanisms underlying statins' protective effect may assist in finding new treatments for ischemic stroke. In this study, we investigated whether the protective effect of statins is mediated by their ability to impact inflammation and oxygen free radical levels in cerebral ischemia/reperfusion injury. Because atorvastatin is a commonly used lipid-lowering drug, we chose it as a representative statin.

The selectins and their ligands are essential for leukocyte rolling and the initiation of the inflammatory response. Produced by endothelial cells after being activated by inflammation, E-selectin plays an important role in the inflammatory cascade after cerebral ischemia. E-selectin is an inducible adhesion molecule that mediates adhesion between endothelial cells and neutrophils, and directly participates in the aggregation and infiltration of inflammatory cells. During cerebral ischemia/reperfusion injury, leukocyte adhesion molecules mediate migration, adhesion and metastasis, to allow white blood cells to migrate across the blood vessel wall and release numerous inflammatory factors. In ischemic brain tissue, a large number of adhesion molecules are expressed[7], and there is an interaction between E-selectin and oxygen free radicals. Early in ischemia, blocking the expression of P-selectin results in a decrease in neutrophil accumulation and adhesion in the ischemic area, thereby reducing neuronal death and infarct volume, and improving outcome. In a variety of animal models of cerebral ischemia, the survival rate increased after anti-E-selectin antibody treatment[8].

Myeloperoxidase exists primarily in neutrophil azurophilic

granules, and its activity is a reliable index of the infiltration of neutrophils in tissue. Myeloperoxidase also plays a crucial role in inflammation. Through the formation of secondary oxidants and nitration, myeloperoxidase regulates intercellular signaling in the vasculature[9]. Myeloperoxidase is specifically expressed in neutrophils and has been used for quantitative determination of neutrophil number, measuring the infiltration of neutrophils, and assessing inflammatory injury.

Both ischemia and inflammation can produce free radicals, and malondialdehyde can cause neuronal injury or death. Malondialdehyde is a stable product of the lipid peroxidation of unsaturated fatty acids by oxygen free radicals. Malondialdehyde can induce DNA and RNA crosslinking, and its levels indirectly reflect changes in oxygen free radical content[10]. Therefore, through the determination of malondialdehyde content, we can estimate oxygen free radical levels in brain tissue and the extent of lipid peroxidation[11]. Free radicals can oxidize unsaturated fatty acids in the membrane and directly damage the blood-brain barrier. Antioxidants, such as superoxide dismutase, can relieve edema in ischemic cerebral tissue and reduce the permeability of the blood-brain barrier[12]. Superoxide dismutase is an antioxidant enzyme that can remove oxygen free radicals and protect against free radical injury. Superoxide dismutase activity is an indirect indicator of the ability to neutralize oxygen free radicals. It has been previously reported that antioxidant enzymes, such as superoxide dismutase and catalase, can inhibit vascular dilation and blood-brain barrier damage caused by hypoxia, thereby attenuating endothelial damage[13].

Statins function as lipid regulators, but they may have other actions in peripheral organs, such as anti-inflammatory effects[14], reducing plaque thrombogenicity, inhibiting cellular proliferation, improving vascular endothelial function[15], and attenuating myocardial reperfusion injury[16]. Statins inhibit the production of cytokines in the endothelium and reduce free radical production in the vascular wall. Accumulating evidence indicates that atorvastatin inhibits the expression of nuclear factor-kB mRNA and protein[17], which are closely associated with inflammation. Statins also provide protection against renal, pulmonary and myocardial ischemia/reperfusion injury[18-20]. However, little evidence is available on similar changes in cerebral ischemia/reperfusion injury. Therefore, we speculated that atorvastatin exerts neuroprotection through its anti-inflammatory and antioxidative effects in acute cerebral ischemia/reperfusion injury. To investigate this possibility, we examined how statins affect inflammation and oxidative stress. This study is characterized by the following key features: (1) The success of the middle cerebral artery occlusion model was determined with triphenyltetrazolium chloride staining; (2) inflammatory factors (E-selectin and myeloperoxidase) and free radicals (superoxide dismutase and malondialdehyde), two important factors in ischemia/reperfusion injury, were assessed; (3) we examined four different time points to observe changes in E-selectin, myeloperoxidase, superoxide dismutase and malondialdehyde; and (4) various methods were used to assess expression.

Our goal was to determine if atorvastatin has a neuroprotective effect, and whether it modulated inflammation and free radical levels. The experiments were performed to gain insight into the therapeutic potential and mechanisms of action of atorvastatin in ischemia/reperfusion injury.

Results

Quantitative analysis of experimental animals

Ninety-six adult rats were divided randomly into three groups: sham surgery group (n = 32; rats only underwent anesthesia and vascular separation), model group (n = 32; middle cerebral artery occlusion model) and atorvastatin group (n = 32; each rat was administered atorvastatin by gavage before middle cerebral artery occlusion). Each group was further divided into four subgroups according to time of sacrifice after cerebral ischemia/reperfusion (4, 8, 12 and 24 hours); each subgroup contained eight animals. Two rats in each subgroup were used for triphenyltetrazolium chloride staining, while the remaining six rats were used for immunohistochemical staining. The sera of all eight rats were used for oxidative stress testing. In the end, 96 rats were included in the analyses.

Triphenyltetrazolium chloride staining for assessing the success of the middle cerebral artery occlusion model

Triphenyltetrazolium chloride staining is one of the most convenient ways to evaluate the success of middle cerebral artery occlusion. Staining was carried out 90 minutes after sham surgery or middle cerebral artery occlusion. Sections in the sham surgery group were uniformly red. Blood supply to the ipsilateral side was significantly reduced, resulting in white regions in the ischemia/reperfusion group, while the contralateral side was red and well-perfused (Figure 1). Thus, the cerebral ischemia/reperfusion model was successfully established.

Effect of atorvastatin on neurological function in rats with cerebral ischemia/reperfusion

Compared with the sham surgery group, the neurological deficit scores in the model and atorvastatin groups were significantly increased 4–24 hours after ischemia/reperfusion (P < 0.05). In addition, the atorvastatin group had a decreased score compared with the model group, with no significant difference (P > 0.05; Figure 2).

Effect of atorvastatin on superoxide dismutase and malondialdehyde content in the serum of rats with cerebral ischemia/reperfusion

The serum specimens in each group were evaluated for superoxide dismutase activity and malondialdehyde content. At each time point after cerebral ischemia/reperfusion, superoxide dismutase activity in the model and atorvastatin groups was significantly lower than in the sham surgery group (P < 0.05), while malondialdehyde content in these two groups was significantly increased (P < 0.05). There were significant differences between the atorvastatin and model groups as well (P < 0.05; Table 1).

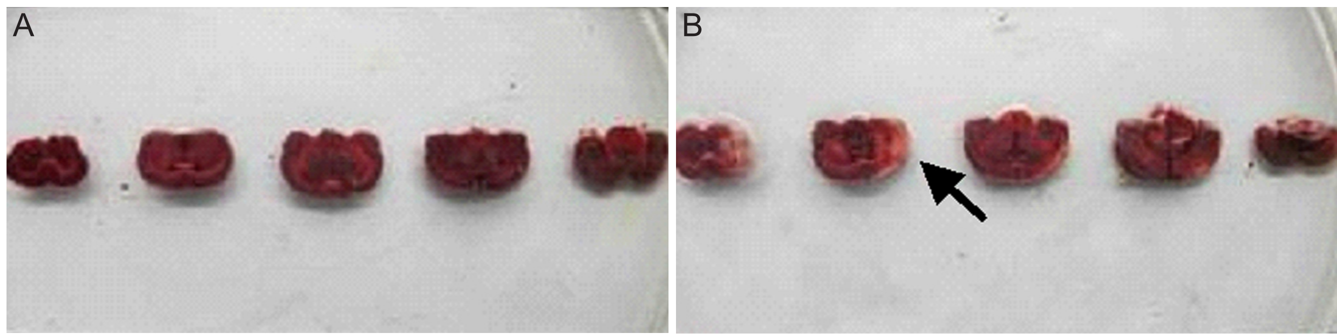

Figure 1 Morphology of the rat brain after middle cerebral artery occlusion (triphenyltetrazolium chloride staining).
In the sham surgery group (A), sections were uniformly red. In the model group (B), the brain tissue in the infarct area was white (arrow).

Table 1 Effect of atorvastatin on superoxide dismutase (SOD) and malondialdehyde (MDA) content in the serum of rats with cerebral ischemia/reperfusion (I/R)

Group	Hours of I/R	SOD (U/mL)	MDA (nmol/mL)
Sham surgery	0	245.3±34.3	2.64±1.28
Model	4	186.8±29.2[a]	5.84±1.48[a]
	8	181.2±12.3[a]	5.90±1.51[a]
	12	177.7±30.2[a]	6.21±2.87[a]
	24	136.4±23.2[a]	9.43±3.98[a]
Atorvastatin	4	201.0±3.2[ab]	4.87±1.21[ab]
	8	193.0±9.0[ab]	5.02±1.97[ab]
	12	189.0±12.9[ab]	6.65±3.25[ab]
	24	184.9±25.2[ab]	6.85±2.32[ab]

Data are expressed as mean ± SD of eight rats for each group. [a]$P < 0.01$, *vs.* sham surgery group; [b]$P < 0.05$, *vs.* model group with the same reperfusion time (one-way analysis of variance and least significant difference test).

Table 2 Effect of atorvastatin on E-selectin expression (gray value) in the cortex of rats with cerebral ischemia/reperfusion (I/R)

	Hours of I/R			
Group	4	8	12	24
Sham surgery	8.44±1.27	12.59±0.80	13.89±1.29	9.85±1.05
Model	28.05±1.28[a]	69.33±0.97[a]	84.19±1.06[a]	60.43±1.33[a]
Atorvastatin	22.32±0.94[ab]	48.20±1.09[ab]	62.93±0.94[ab]	41.47±1.18[ab]

Data are presented as the average gray value (calculated by MIAS medical image analysis system) of the immunohistochemical staining image. The higher average gray value represents higher protein expression. [a]$P < 0.05$, *vs.* sham surgery group; [b]$P < 0.05$, *vs.* model group. Data are expressed as mean ± SD of six rats for each group (one-way analysis of variance and least significant difference test).

Effect of atorvastatin on E-selectin and myeloperoxidase expression in the cortex of rats with cerebral ischemia/reperfusion

After 90 minutes of middle cerebral artery occlusion or sham surgery, E-selectin expression in the ischemic brain tissue was determined with immunohistochemical staining at 4, 8, 12 and 24 hours after reperfusion. Expression of E-selectin and myeloperoxidase in the endothelial cells was observed as tan-yellow staining in the cell boundaries and cytoplasm. The average gray value of positive cells in each

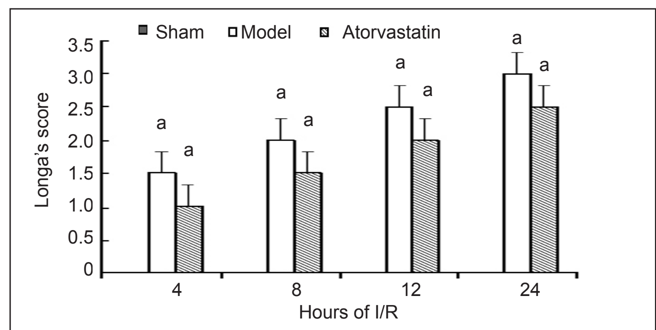

Figure 2 Effect of atorvastatin on neurological function in rats with cerebral ischemia/reperfusion (I/R).
Data are expressed as mean ± SD of eight rats for each group. [a]$P < 0.05$, *vs.* sham group (0 score) (one-way analysis of variance and least significant difference test). The higher scores indicate more severe neurological function deficit. Sham: Sham surgery group.

group at each time point was measured (the average gray value was positively correlated with protein expression). There was weak expression of E-selectin and myeloperoxidase in the cortex of sham-operated rats, indicating that E-selectin and myeloperoxidase are scarcely expressed in normal brain tissue. Compared with the sham surgery group, E-selectin and myeloperoxidase expression was significantly increased in the model and atorvastatin groups ($P < 0.05$). The expression levels in the atorvastatin group were significantly lower than in the model group ($P < 0.05$). The expression peaked 12 hours after cerebral ischemia/reperfusion (Figures 3, 4; Tables 2, 3).

Discussion

Acute ischemic cerebrovascular disease is a serious threat to human health because it is associated with high disability and death. Thus, active prevention and early effective treatment are essential. Oxygen free radicals promote neuronal and blood-brain barrier damage following ischemia/reperfusion. The blood-brain barrier microvasculature is vulnerable to oxidative damage due to a relatively low antioxidant capacity, high membrane polyunsaturated fatty acid content and accessibility to redox active iron[21]. Because blood-brain barrier damage brings about serious brain edema, hemorrhage and high mortality in acute ischemic stroke, the potential aggravation of blood-brain barrier damage by oxygen free radicals is a major problem.

In this study, triphenyltetrazolium chloride staining

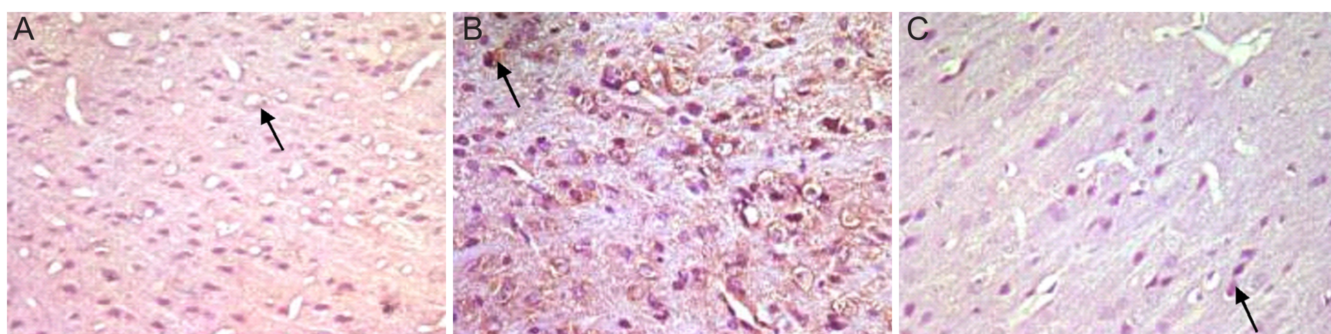

Figure 3 Effect of atorvastatin on E-selectin expression in the cortex of rats with cerebral ischemia/reperfusion (immunohistochemical staining, × 400).
E-selectin-positive cells (arrows) were mainly distributed in the cortex and hippocampus, and E-selectin immunoreactivity was expressed in nerve cells and vascular endothelial cell membranes and cytoplasm.
E-selectin expression in the model group (B) and in the atorvastatin group (C) was increased significantly compared with the sham surgery group (A). Compared with the model group, the increase in E-selectin expression was significantly inhibited in the atorvastatin group. The arrows show expression of E-selectin.

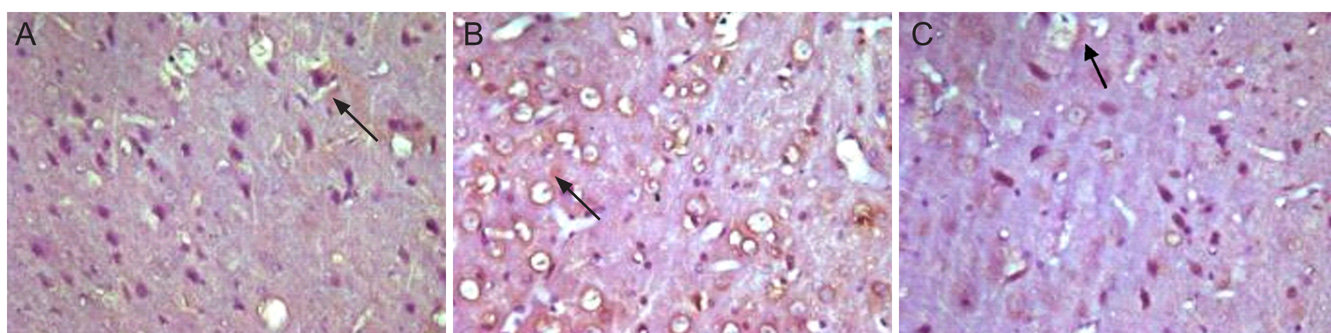

Figure 4 Effect of atorvastatin on myeloperoxidase expression in the cortex of rats with cerebral ischemia/reperfusion (immunohistochemical staining, × 400).
Myeloperoxidase labeling (arrows) was mainly distributed in the cytoplasm, with minor labeling in the cell membrane. Myeloperoxidase expression in the model and atorvastatin groups was increased significantly compared with the sham surgery group (A). Compared with the model group (B), the increase in myeloperoxidase expression was significantly inhibited in the atorvastatin group (C). Arrows point to labeling for myeloperoxidase.

Table 3 Effect of atorvastatin on myeloperoxidase expression (gray value) in the cortex of rats with cerebral ischemia/reperfusion

	Reperfusion time (hour)			
Group	4	8	12	24
Sham surgery	7.98±1.05	7.79±1.26	8.22±0.89	7.51±1.17
Model	15.95±2.00[a]	20.45±1.97[a]	21.75±2.61[a]	18.10±0.49[a]
Atorvastatin	12.55±1.51[ab]	13.80±0.44[ab]	15.14±0.36[ab]	11.84±0.33[ab]

Data are presented as the average gray value of the immunohistochemical staining image. Average gray values were analyzed by MIAS medical image analysis system. The higher average gray value represents higher protein expression. Myeloperoxidase expression in the model group and the atorvastatin group was increased significantly compared with the sham surgery group. Compared with the model group, the increase in myeloperoxidase expression was significantly inhibited in the atorvastatin group. [a]P < 0.05, *vs.* sham surgery group; [b]P < 0.05, *vs.* model group. Data are expressed as mean ± SD of six rats for each group (one-way analysis of variance and least significant difference test).

showed that sections in the sham surgery group were uniformly red. Blood supply to the ipsilateral brain was significantly reduced in the ischemia/reperfusion group, while the contralateral side was red and well-perfused, as previously described[22-23]. Therefore, triphenyltetrazolium chloride staining can be used to determine whether the middle cerebral artery occlusion model was established successfully. This model was chosen to study the relationship between inflammatory factors and oxygen free radicals in brain tissues after cerebral ischemia/reperfusion injury, and to investigate the neuroprotective mechanisms of atorvastatin.

Free radicals and inflammation play major roles in tissue damage after ischemia/reperfusion injury[24-25]. As an important mediator of damage to nerve cells in ischemic brain tissue and to the blood-brain barrier, oxygen free radicals may exacerbate the occurrence and development of brain edema, post-ischemic hemorrhage and tissue necrosis after ischemic stroke. Oxygen supply and ATP levels are decreased during cerebral ischemia, which causes excess production of hydrogen peroxide and other oxygen-derived free radicals[26]. Concomitantly, the dynamic equilibrium between superoxide dismutase and superoxide is impaired. Free radicals attack membrane structures and DNA, damaging endothelial cell membranes and leading to impairments in ion transport, energy production and cell function.

Our findings in this study showed that malondialdehyde content in the model and atorvastatin groups at each time point increased. Malondialdehyde is the end product of lipid peroxidation, and its content is an indicator of lipid peroxidation *in vivo*, reflecting free radical concentration[15]. Our result

is consistent with previous studies[27-28], showing that cerebral ischemia causes an increase in free radicals and a decrease in superoxide dismutase activity. Superoxide dismutase inhibits vascular dilation and blood-brain barrier damage caused by hypoxia, and can reduce endothelial damage. Both malondialdehyde content and superoxide dismutase activity change with time, and may parallel neurologic impairment.

In this study, superoxide dismutase activity in the atorvastatin group was significantly lower than in the model group at each time point, but higher than in the sham surgery group. Malondialdehyde content in the atorvastatin group was significantly higher than in the model group at each time point, but lower than in the sham surgery group. We speculate that cerebral ischemia causes excess production of hydrogen peroxide and other free radicals and impairs superoxide dismutase activity. Atorvastatin pretreatment before cerebral ischemia decreases free radical production and increases the ability to neutralize free radicals, but does not completely inhibit free radical damage caused by ischemia. This is concordant with the neurological deficit scores found in this study. Atorvastatin improved superoxide dismutase activity and reduced malondialdehyde content, suggesting that it can protect superoxide dismutase and that it has antioxidant properties, reducing the generation of lipid peroxides. Because the animal model in this study had normal blood lipid levels, and the drug delivery regimen was short, the antioxidant function of atorvastatin cannot be explained by its cholesterol-lowering action. Therefore, we conclude that atorvastatin might exert neuroprotection by lowering levels of free radicals produced after ischemia/reperfusion.

Growing evidence highlights the contribution of the inflammatory response in cerebral ischemia/reperfusion injury. Reducing inflammation significantly decreases cerebral ischemia/reperfusion injury. Both free radicals and ischemia can induce an inflammatory response. It was recently found that excessive inflammation in cerebral tissue after cerebral ischemia/reperfusion is one of the main causes of reperfusion injury[29]. Acute inflammatory reactions after ischemia/reperfusion accelerate secondary cerebral injury[30-32]. Inflammation is characterized by the activation of vascular endothelial cells and increases in adhesion molecules on the surface of leukocytes, which promotes obstruction of microvessels and leukocyte infiltration into the brain parenchyma, where they enhance reactive oxygen species production and tissue damage[33-34]. Clearing peripheral leukocytes or inhibiting their activity with drugs can protect cerebral tissue from ischemia/reperfusion injury[35].

In cerebral ischemia/reperfusion, white blood cells and endothelial cells interact through E-selectin. E-selectin expression on the surface of endothelial cells increases when they are stimulated by inflammation[36]. In this study, immunohistochemistry showed that E-selectin was expressed in the ischemic ipsilateral cortex, striatum and hippocampus, and there were only a few E-selectin-positive cells in the sham surgery group, which is similar to previous reports[37-38]. Compared with the sham surgery group, there were more positive cells in the cortex and hippocampus after cerebral ischemia/reperfusion. E-selectin expression changed dynamically, starting at 4 hours, peaking at 12 hours and declining by 24 hours. This finding is similar to previous reports[39-42]. Blood-exposed vessels show a significant increase in intercellular adhesion molecule-1 immunoreactivity, peaking at 24 hours after blood exposure and reaching the baseline again by 48 hours[39]. In a primate stroke model, antibody treatment with anti-adhesion molecule receptors can reduce the "non circulating flow" phenomenon[43]. In Sprague-Dawley rats with knockout of both CD18 and CD26E, the adhesion of leukocytes fell by 95%[44]. Previous studies suggest that the effects of E-selectin are specific and cannot be replaced by any other adhesion molecule[38]. E-selectin antibody dramatically reduces adhesion and infiltration of leukocytes, leading to an increase in blood flow in the ischemic region and a decrease in infarct area[45].

Could the change in E-selectin after cerebral ischemia/reperfusion correlate with the severity of inflammation? Myeloperoxidase is a peroxidase enzyme encoded by the myeloperoxidase gene. Myeloperoxidase is abundantly expressed in neutrophil granulocytes (a subtype of white blood cells)[46-47] and is stored in azurophilic granules, and is the most reliable biomarker of the infiltration of neutrophils in tissue[48-50]. In this experiment, myeloperoxidase was expressed in the ischemic ipsilateral cortex, striatum and hippocampus, and there were few myeloperoxidase positive expressions at each time point in the sham surgery group, which is similar to previous reports[37-38]. Compared with the sham surgery group, there were more positive cells in the cortex and hippocampus after cerebral ischemia/reperfusion. Myeloperoxidase expression changed dynamically, starting at 4 hours, peaking at 12 hours and remaining at 24 hours, but with significant reduction. This temporal pattern is similar to the changes in E-selectin expression and corresponds with the severity of neurological deficit. Therefore, we speculate that E-selectin is associated with the degree of inflammation.

Our findings show that biomarkers of inflammation and oxidative injury change over time; thus, we conjectured that cerebral ischemia reperfusion causes disorders in both the antioxidant and inflammatory systems, but it remains unclear whether free radicals or inflammatory factors are the initiators in this reaction chain. However, we believe that both free radical damage and inflammatory injury are two main types of damage in cerebral ischemia/reperfusion. Atorvastatin, one of the statins, has relatively better antioxidant activity[51] and has been used to treat ischemic stroke[52-53]. Statins can not only reduce low-density lipoprotein levels, but can also increase high-density lipoprotein. In addition, statins help improve endothelial function, influencing the structure and stability of plaques and preventing thrombosis. Mueck et al.[54] found that fluvastatin decreases expression of intercellular adhesion molecule 1, vascular cell adhesion molecule 1 and E-selectin in a dose-dependent manner.

In this study, compared with the model group, E-selectin expression in the atorvastatin group was significantly lower at each time point, but higher than in the sham surgery group. This evidence suggests that atorvastatin pretreatment before cerebral ischemia could decrease inflammation, but could not inhibit damage completely. For further confirmation, myeloperoxidase levels were compared among the three different groups. The results showed that, compared with the model group, myeloperoxidase expression in the atorvastatin group was significantly lower at each time point, but higher than in the sham surgery group. This evidence suggests that atorvastatin pretreatment before cerebral ischemia can decrease, but not fully inhibit, myeloperoxidase expression

during ischemia.

It is unclear how atorvastatin reduces E-selectin expression or provides neuroprotection. With its anti-inflammatory effects[55-56], atorvastatin can lower the levels of total cholesterol, LDC-C and monocyte chemotactic protein-1, and increase low-density lipoprotein[57]. High-density lipoprotein was shown to inhibit E-selectin expression[58]. E-selectin expression in human umbilical vein and in L1-overexpressing pig aortic endothelial cells is inhibited by high-density lipoprotein[59]. Therefore, we hypothesize that atorvastatin may reduce E-selectin expression by raising the level of high-density lipoprotein, thereby contributing to cerebral protection.

Our findings indicate that atorvastatin inhibits E-selectin and myeloperoxidase expression after cerebral ischemia/reperfusion. Atorvastatin likely inhibits E-selectin gene expression, thereby reducing levels of the protein. Atorvastatin also inhibits the adhesion and infiltration of leukocytes and endothelial cells, thus contributing to cerebral protection by reducing inflammatory damage after cerebral ischemia/reperfusion. Furthermore, atorvastatin can decrease free radical generation and enhance antioxidant activity. Free radicals and proinflammatory factors work in combination to promote injury in ischemia. Therefore, atorvastatin is a promising neuroprotective agent in cerebral ischemia/reperfusion injury.

In summary, in cerebral ischemia/reperfusion injury, oxidative injury and inflammatory response are induced, and the administration of atorvastatin protects against injury. Atorvastatin improves lipid parameters and decreases cerebral lipid peroxidation and inflammation, and it increases levels of the antioxidant superoxide dismutase. Together, our findings suggest that statins can have significant benefits for patients with normal blood lipid levels. Further study is required to fully clarify the neuroprotective effects of atorvastatin, and future experiments should examine dose-effect relationships to more clearly establish the therapeutic potential of statins in the treatment of ischemic brain injury.

Materials and Methods

Design

A completely randomized animal experiment.

Time and setting

The experiment was performed in the Central Laboratory of the Second Xiangya Hospital of Central South University, China, from July 2011 to February 2012.

Materials

Ninety-six Sprague-Dawley adult rats weighing 240–260 g were provided by the Laboratory Animal Center of Xiangya Medical School of Central South University, China (license No. SYXK (Xiang) 2012-003). Experimental procedures on animals were in strict accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[60].

Methods

Establishing middle cerebral ischemia/reperfusion model

A model of focal cerebral ischemia/reperfusion was established in Sprague-Dawley rats according to the Longa suture method[61]. A silicone-coated nylon monofilament was passed through the bifurcation of the common carotid artery to the internal carotid artery, then into the brain and advanced along the internal carotid artery to approximately 18–22 mm from the bifurcation until a proximal occlusion of the right middle cerebral artery was established. After 90 minutes of occlusion, the filament was withdrawn slowly to recover blood supply in the middle cerebral artery for 24 hours of reperfusion. Room temperature was maintained at 25°C during the operation. The body temperature was maintained at 37°C. A blood sample was drawn for testing superoxide dismutase and malondialdehyde content. The success of the model was evaluated using neurological deficit scoring and triphenyltetrazolium chloride staining.

Atorvastatin treatment

Each rat in the atorvastatin group received a daily dose of 6.5 mg/kg atorvastatin starting 1 week before the operation. Atorvastatin was dissolved in 0.9% normal saline and administered by gavage. The other groups were given a gavage of normal saline of the same volume.

Neurological deficit scoring

After recovery from anesthesia, neurological symptoms were observed before further experimentation. Neurological deficit scoring was performed according to Longa's method[62]. Scoring was as follows: 0, the rat demonstrated no symptoms of neural damage; 1, the rat could not fully extend the forepaws; 2, the rat circled to the opposite side; 3, the rat fell down the opposite side; 4, the rat could not walk spontaneously, with loss of consciousness. Rats receiving a score of 1–3 were selected for further evaluation.

Processing of brain tissue and slice preparation

Anesthesia was achieved with 10% novochlorhydrate (350 mg/kg). After perfusion, the animal was decapitated and brain tissue was removed. Then, the fixed brain tissue was serially cut into 4-μm coronary sections between the optic chiasm and the leading edge of the pons, followed by conventional dehydration, clearing, wax-soaking and embedding. Rats were excluded if subarachnoid hemorrhage occurred in the skull base.

Triphenyltetrazolium chloride staining for assessing the success of middle cerebral artery occlusionrocessing

After anesthesia with 10% novochlorhydrate, the brain tissue was harvested and refrigerated at −20°C for 30 minutes. Then, five coronal slices were cut with an interval of 2-mm behind the frontal pole (at 1 mm, 3 mm, 5 mm, 7 mm, 9 mm, 11 mm) and incubated in the dark for 30 minutes with 2% triphenyltetrazolium chloride-PBS solution at 37°C. Regions that stained red were normal, and regions of cerebral infarction appeared white. After staining, sections were fixed with 10% formaldehyde solution for 24 hours and photographed with a digital camera (Canon, Tokyo, Japan).

E-selectin and myeloperoxidase expression as detected by immunohistochemical staining

After dewaxing and hydration, brain tissue paraffin sections were subjected to heat-induced epitope retrieval, then

washed with PBS three times for 5 minutes each. Then, each section was blocked with 50 μL peroxidase blocking solution, to block endogenous peroxidase activity, for 10 minutes at room temperature. After three PBS washes, each section was incubated with 50 μL normal serum (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) for 10 minutes at room temperature prior to overnight incubation at 4°C with 50 μL rabbit anti-mouse E-selectin monoclonal antibody or rabbit anti-mouse myeloperoxidase polyclonal antibody (Beijing Biosynthesis Biotechnology). After three PBS washings, each section was incubated with 50 μL biotin-labeled goat anti-rat IgG (Beijing Biosynthesis Biotechnology) and 50 μL streptomyces avidin peroxidase solution for 10 minutes at room temperature. Then, each section was observed with a microscope for 10 minutes following the addition of 100 μL freshly prepared AEC solution, resulting in a brownish reaction product.

The immunohistochemical testing results were processed by computer image analysis system. The gray value was measured using MIAS medical image analysis system (BeiHang Company, Beijing, China), and the average grey value was selected for assessing E-selectin and myeloperoxidase expression in each section. Polyclonal rabbit anti-mouse E-selectin antibodies (1:100), polyclonal rabbit anti-mouse myeloperoxidase antibodies (1:100) and biotin-labeled sheep anti-rabbit IgG (1:100) were purchased from Biosynthesis Biotechnology Co., Ltd. Immunohistochemical staining protocols (streptavidin-alkaline phosphatase method) and diaminobenzidine coloration were used for amplification and visualization. Data are represented as the average gray of immunohistochemical staining images.

Superoxide dismutase and malondialdehyde content in serum

At the end of the experimental period, the rats in each group were deprived of food overnight, but allowed free access to water, and then sacrificed. The blood was collected by retro-orbital puncture, and serum was separated. Serum was analyzed using a malondialdehyde kit (Shanghai Bangyi Trading Co., Ltd., Shanghai, China) and a superoxide dismutase kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China). Superoxide dismutase activity and malondialdehyde content in serum were quantitated spectrophotometrically (Shanghai TianPu Analysis Instrument Co., Ltd., Shanghai, China).

Statistical analysis

Data are expressed as mean ± SD and were analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Differences between groups were compared by one-way analysis of variance and least significant difference test. A $P < 0.05$ was considered statistically significant.

Author contributions: *Tang XQ was responsible for the study concept and design, was the head of the fund, gained the article approval, presided over the overall implementation of the experiment. Tu QY participated in the study guidance and provided immunohistochemical technical support. Zhong W performed data analysis. Cao H implemented animal experiments and performed data analysis. Ding BR was responsible for animal experiments and statistical processing. All authors approved the final version of the manuscript.*

Conflicts of interest: *None declared.*

Peer review: *This study aims to observe the potential protective effect of atorvastatin in cerebral ischemia/reperfusion injury rats through observing the change of inflammation and oxidative stress parameters, in an effort to speculate that atorvastatin benefits the therapeutic effect of cerebrovascular disease patients at normal blood lipid level, indicating great clinical value.*

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