The Protective Effects of Atorvastatin on the Brain Injury and Neuronal Damage caused by Ischemia-reperfusion in Rats

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ABSTRACT

BACKGROUND AND OBJECTIVE: Stroke is the third main cause of mortality and the most important origin of disability in the world. Atorvastatin has been shown to have strong anti-inflammatory and antioxidant effects on various pathological conditions. This study aimed to investigate the possible protective effects of atorvastatin on neuronal injury and damage in an experimental model of focal cerebral ischemia in rats.

METHODS: This experimental study was conducted on 32 male Wistar rats, divided in four groups of eight: the control group, ischemia control group, and ischemia treated with atorvastatin (pre-treatment and post-treatment groups). Cerebral ischemia was determined as 90 minutes of middle cerebral artery occlusion, and 24 hours of reperfusion. Atorvastatin (40 mg/kg) was injected intraperitoneally one hour before ischemia induction, and immediately after the initiation of reperfusion. Moreover, evaluation of motor neuron disorders (NDS index), lesion volumes (TTC dyes) and damage recognition were performed 24 hours after the occlusion of the middle cerebral artery.

FINDINGS: In this study, the occlusion of middle cerebral artery caused significant lesions in the right hemisphere of the rats in the ischemia control group (483±69 mm³), with NDS of 3.20±0.20. Use of atorvastatin in the pre- and post-treatment ischemia groups significantly reduced lesion volumes to 68% and 54%, respectively (P<0.05). In addition, NDS reduced by 25% in both treatment groups (p<0.05). In histological investigations, atorvastatin significantly decreased the number of damaged neurons and eosinophilia, as well as the demyelination of axons and destruction of nerve tissues in the ischemic areas of the brain.

CONCLUSION: According to the results of this study, atorvastatin could effectively reduce the brain damage caused by ischemia-reperfusion, while preventing neuronal destruction and pathological changes of the brain during ischemia.

KEY WORDS: Atorvastatin, Ischemia-Reperfusion, Neuroprotective, Brain Injury, Pathological Changes.

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Introduction

After cancer and cardiovascular diseases, stroke is the most important cause of mortality in the world (1). Although the prevalence of strokes has declined because of the recent advances in medical science, this disorder is still of great consequence among the neurological diseases of adults in terms of prevalence, and in addition to death, it could lead to severe disabilities in 50% of the survivors (2).

The main regions of stroke are the central compartment (Core) and the environmental compartment (Penumbra) of the brain. If the core is damaged by the stroke, the cells are prone to injury and could be destroyed within the initial moments due to the severe reduction or cessation of blood flow. On the other hand, the brain cells within the penumbra are likely to survive longer due to receiving limited nutrition from ancillary vessels; however, they may lose their ability to function properly (3).

Cerebral ischemic injuries are normally caused by a complex series of pathophysiological factors, such as the reduction of oxygen, nutritional requirements of cells, removal of harmful cellular wastes, and the production of toxic materials (4). These factors could result in the uncontrollable depolarization of neurons, excitatory amino-acid release, increased number of cytokines and inflammatory markers, production of free radicals, acidosis and elevation of intracellular calcium levels. Furthermore, these factors could induce neuronal death and injury expansion after ischemia and stroke (5, 6). Although reperfusion in ischemic areas results in oxygen transfer, as well as the provision of metabolic requirements for cells and removal of cellular wastes, in some cases, increased production of oxygen free radicals, nitric oxide and intracellular calcium might intensify ischemic damages (7).

Despite the remarkable advances in the pathophysiology of strokes, there are limited treatment options. Recently, recombinant tissue plasminogen activators (rt-IPAs) have been shown to be effective in the treatment of ischemia and stroke damages; however, the use of this medication is still restricted due to the short-term stroke treatment window, as well as the possible complications caused by the risk of hemorrhage (8).

Therefore, identification of new drugs in order to reduce brain injury and other side effects of stroke is of paramount importance, and the use of Statins has recently been considered as a treatment option by several researchers. Statins are competitive enzyme inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which could reduce the amount of blood cholesterol through hepatic enzyme inhibition. Recent findings have reported this group of drugs to have remarkable effects on the prevention of cardiovascular complications in pathological conditions, such as diabetes mellitus and arterial hypertension, apart from their cholesterol-reducing functions (9-11).

Furthermore, other studies have indicated that in addition to decreasing the level of blood cholesterol and triglyceride hypoglycemic, these drugs have anti-inflammatory, vasodilatory, antioxidant and anti-apoptotic properties (12, 13).

Scientific findings during the past decade have also confirmed the desirable effects of statins on the prevention of severe injuries caused by strokes. Accordingly, statins are one of the most practical combinations for the prevention and treatment of coronary artery diseases. In addition, the use of these medications in patients with acute coronary incidents, even in those without high levels of serum lipids, has been shown to have positive effects on the treatment of these patients (14).

In one study, Sever et al. demonstrated that atorvastatin was able to prevent the incidence of strokes or cardiovascular episodes in patients with hypertension, and average or low cholesterol levels (15). In another study by Kilic et al., the intraperitoneal injection of statins (20 mg/kg) in the model of ischemia, induced by middle cerebral artery ligation in mice, was observed to reduce the severity of the brain injury caused by ischemia (16).

In another study by Wang et al., the use of atorvastatin, accompanied by Sildenafil, was reported to be effective in reducing the side effects of strokes induced in mice (17). Similarly, Kawai et al. demonstrated that the use of Amlodipine and atorvastatin could exert remarkable neuroprotective effects on a model of experimental ischemia in Zucker metabolic rats (18). Moreover, Wang et al. reported the use of Simvastatin to have beneficial effects on the subsequent traumatic brain injury, resulting in the improvement of the local cerebral blood flow, reduced destruction of hippocampus neurons, and better cognitive and behavioral outcomes (19).

Based on the results of the aforementioned studies, as well as the mechanism of injury and pathophysiology of strokes, it could be concluded that use of statins could be an effective approach as to diminish the subsequent
effects of strokes. The present study aimed to investigate the protective effects of atorvastatin, with an antioxidant and anti-inflammatory origin, on the reduction of the brain damage caused by ischemia-reperfusion, as well as the pathological changes in the brain of rats during stroke. This study was conducted on an experimental model of local and transient ischemia.

Methods

Animals and the Method of Preservation: This study was conducted on 32 male Wistar rats (weight: 270-320 g), which were purchased from the animal laboratory of Baqiyatallah (AJ) University of Medical Sciences. Before the study and during the tests, the animals were maintained within a photocycle of 12 hours of light and 12 hours of darkness in the ideal humidity and temperature of 25°C, with free access to food and water. All the experiments were performed in accordance with the ethics of animal testing, and the study protocol was approved by the Research Council of Baqiyatallah University of Medical Sciences.

Induction of Transient Focal Cerebral Ischemia: In order to prepare the animals for temporary focal ischemia, we used the method proposed by Longa et al. (20). Initially, the rats were anesthetized using 2.5% Isoflurane (Isoflurane, UK). After stabilizing the animals on a surgical table, the connective and muscle tissues of the neck were sliced aside for easy access to the common carotid artery and the internal and external branches.

In the ischemic groups, by the temporary closing of the right common carotid artery, a subtle incision was made in the branches of the external carotid. Simultaneously, a nylon thread was prepared by tying the lower down section (No. 0-3), the tip of which was heated and the surface was covered with poly-L-lysine. Afterwards, the thread was placed into the artery and guided slowly into the skull and the circle of Willis until reaching the middle cerebral artery. By passing the tip of the thread through the middle cerebral artery, a slight resistance was induced in the direction of the nylon thread (21).

After ascertaining the correct placement of the nylon thread in the determined spot, it was stabilized in place. In order to terminate the ischemia and reperfusion, the nylon thread was slowly removed from the vein, and by tying the external artery, the scars in the neck area were stitched, and the animal was maintained in warm conditions until regaining consciousness.

During all the experiments until the termination of anesthesia, the body temperature of the animals was stabilized between 38-37°C using a heat lamp. After regaining full consciousness, the animals were transferred to cages and kept under proper maintenance for further experiments.

The Study Protocol and Experimental Groups: In this study, the animals were randomly divided into four groups, which are as follows:

The Control Group (Sham, N=8): In this group, surgery was performed on the animals after anesthesia, with the detection of common carotid artery, as well as the internal and external carotid arteries in the neck, in order to induce middle cerebral artery occlusion. However, the occlusion of the middle cerebral artery could not be performed. As a result, due to the absence of middle cerebral artery occlusion, no signs of cerebral ischemia were observed after the preparation and staining of the obtained brain sections.

Ischemia-reperfusion Group (I-R, N=8): The animals in this group underwent similar operations to the control group until the induction of ischemia. Ischemia induction was sustained for 90 minutes, and the reperfusion stage was initiated with the slow removal of the nylon thread, and the surgery spot was stitched after 15 minutes; afterwards, the animal was preserved in proper conditions. The evaluation of movement disorders was carried out 24 hours after the initiation of reperfusion in the surviving animals. Finally, the animals were killed with the intraperitoneal injection of thiopental sodium under anesthesia, and the brains were carefully removed from the scalp and prepared for the investigation of lesion volumes.

Pre-treatment Group with Atorvastatin (I-R+Pre, N=8): The animals in this group received intraperitoneal injection of atorvastatin (40 mg/kg) one hour before the induction of ischemia, and the rest of the protocols and operations were carried out within the same procedure as the I-R group for filament investments to induce ischemia-reperfusion.

Post-treatment Group with Atorvastatin (I-R+Post, N=8): The animals in this group were intraperitoneally administered with atorvastatin (40 mg/kg) at the end of middle cerebral artery occlusion, and immediately at the onset of reperfusion. The rest of the protocols and operations for filament investments were carried out similar to the I-R group in order to induce ischemia-reperfusion. For this study, atorvastatin was purchased from Hakim Pharmaceutical Co. (Iran). In order to prepare atorvastatin for injection, the powder of the
drug was initially weighed using a digital scale, and it was freshly resolyed in normal saline. Following that, the atorvastatin solution was injected intraperitoneally to each animal after measuring the precise amount for injection (40 mg/kg). The injection volume was determined between 5.0-1 ml based on the weight of the animals and further calculations. In addition, an equivalent amount of saline was injected to the animals in the control and ischemia-control groups.

Assessment of Motor Neuron Disorders: In the animals that survived within 24 hours after the end of ischemia, the motor neuron disorders were evaluated using a five-point test by a person who had no information about the treatment group (22), and the movement disorders of the animals were scored from 1 to 5. The animals that showed no symptoms of movement disorders were scored 1, including the rats in the control group. The animals who would bend the opposite hand to the ischemic sphere (i.e. left hand) from the shoulder during suspension (i.e. Flexion mode) were scored 2. Score 3 was given to the animals that would turn to the opposite side of the ischemic hemisphere (i.e. left side) at the beginning of movement on a surface. In addition, the animals that had lost the righting reflex were scored 4, and score 5 was considered for the animals that had no ability of conscious movement.

Measurement of Brain Lesion Volumes: In order to measure the volume of brain lesions in the ischemic hemispheres of the studied animals, the extracted brains were placed in normal saline (4°C) for 5 minutes to harden. Afterwards, six coronal cross cuts (diameter of 2 mm) were prepared using the Brain Metrix. For staining, the cuts were laid in a solution of 2% triphenyltetrazolium chloride (TTC) for 20 minutes. Via this method, the ischemic parts of the brain would become white, while the healthy spots would turn brick red. The dyed sections were placed in 10% buffered formalin for fixture, and for the final preparation, each of the sections was photographed separately by a digital camera (Cannon, Japan). Moreover, NIH image processing was used to evaluate the damaged areas of the cortex and striatum (22).

The volume of the lesions in the cortex and striatum were added for all sections in cubic millimeters (mm³), and the amount of revised lesions (corrected infarction volume) was calculated based on the following formula: Corrected infarction volume (mm³) = [non-ischemic hemisphere volume - (volume of ischemic hemisphere - calculated lesion volume)]

Measurement of Tissue Swelling: In order to measure the percentage of tissue swelling, the volume of lesions in the right hemisphere (RH) and the healthy left hemisphere (LH) were calculated in mm³ in the stained sections, and the tissue swelling was measured based on the following formula (22):

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\text{healthy hemisphere - injured hemisphere} \times 100 = \text{Tissue swelling}
\]

Investigation of Pathological Changes in the Ischemic Section of the Brain: In this study, the animals in the control and ischemic groups (i.e. control-ischemia and treated ischemia) were anesthetized by ether 24 hours after the reperfusion. For microscopic studies, brain tissue samples were placed in 10% formalin for two weeks to stabilize. The experiments proceeded by tissue preparation, and casting was also carried out using paraffin according to the routine procedures. Afterwards, the samples were cut in diameters of 5 mm using a microtome.

The prepared sections were placed on a slide and stained using the method of Hematoxylin and Eosin (H & E) stain, and Luxol Fast Blue (LFB) stain. Following that, the prepared sections were dehydrated and clarified, and the mount process was carried out as well. Finally, the slides were carefully examined with optical microscope (Nikon), and a special camera attached to the microscope and computer (CMEX) was used to photograph the determined spots. Moreover, indicators of necrosis and neuronal injury (i.e. triangular acidophil neurons with wrinkled, dense cores) were evaluated, as well as the destruction level of the nerve tissues (i.e. vacuolization).

Statistical Analysis: The obtained results of this study were presented as mean and Standard Error (Mean ± SE). To compare the volume of lesions between the study groups, one-way ANOVA and Tukey’s test were used. In addition, to compare the obtained data of movement disorders, non-parametric tests and Mann-Whitney U test were used, and p<0.05 was considered as significant.

Result

The Results of Movement Disorders: In the control group, no signs of motor neuron disorders were observed; therefore, the rate of this parameter was equal to one in this group. The measured value for these disorders was 3.20±0.20 out of 5 in the ischemia-control
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Furthermore, treatment with atorvastatin significantly decreased the severity of movement disorders in the pre-treatment and post-treatment groups (p<0.05). The rate of this parameter was 2.40±0.24 in the pre-treatment group and 2.20±0.24 in the post-treatment group (out of 5). No statistically significant difference was observed between the two groups in this regard (fig 1).

The Results of Brain Lesion Volume Measurements:
The uniformity of the brick red color confirmed the absence of tissue damage in the right and left hemispheres of the control groups, as well as the left hemispheres of the rats in the treatment and non-treated ischemic groups. Nevertheless, induction of ischemia-reperfusion was found to cause a relatively large injury with a white color in the cortical and subcortical regions of the right hemispheres of the animals in the ischemic groups (fig 2).

As depicted in the images, the volume of the lesions significantly decreased in both treatment groups before and after ischemia, compared to the ischemic control group. The total lesion volume in the cortical and subcortical regions of the ischemic hemispheres was 483±69 in the animals of the ischemic group (fig 3). Furthermore, a significant reduction was observed in the total volume of lesions after the administration of atorvastatin (40 mg/kg) one hour before the induction of ischemia, and immediately after reperfusion.

The total volume of lesions in the pre-treatment group was 218±37 mm³ (p<0.05), while it was 153±28 mm³ in the post-treatment group (p<0.01). No significant difference was observed between the two treatment groups in this regard.

The Results of Tissue Swelling Measurements: In this study, the percentage of right hemisphere edema in the control group, which was calculated by the volume of hemispheres, was close to zero, while in the pre-treatment and post-treatment groups, this volume had a significant increase (14.76±2.34%) (fig 4). Furthermore, the level of edema in the pre-treatment and post-treatment groups was 7.63±1.76% and 7.52±0.52%, respectively, which had a significant reduction compared to the ischemic-control group (p<0.01).

Pathological Findings: The sections of the right hemisphere cortex, which were under middle cerebral artery perfusion, were stained using the H & E method. The neurons and other cells of this area, as well as the nerve tissues, were observed to be normal in the control.
These cells form coherent tissues, which are located in the parenchyma tissue of the brain (i.e. neuropil). In this study, the investigation of the sections provided from the animals in the ischemic-control group revealed considerable neuronal damage, as well as the vacuolization of neuronal cortex tissue, and the high density of the cores were indicative of the presence of necrotic and pyknotic neurons (I-R) (fig 5). However, the administration of atorvastatin before and after ischemia was able to prevent the aforementioned injuries in the animals of the treatment groups, and the conditions of neurons and nerve tissues were observed to be normal in both the treatment and control groups (Pre- and Post-treatment) (fig 6).

**Figure 4. Volume of Tissue Swelling (%), 24 hours after Reperfusion in Treatment Groups (Data presented as Mean±SE)**

"Significant Difference with Ischemic-reperfusion Control Group (I-R) (p<0.01)

**Figure 5. Optical Microscopic Images of Cross Sections of Right Hemisphere Cortex, 24 hours after Reperfusion (Stained via H & E)**

In the captured images of the cross sections provided from the animals in the control group (S), healthy neurons and nerve tissues were detected, whereas in the I-R group, damaged neurons with dense and wrinkled cores (i.e. pyknotic and necrotic neurons) were observed, as well as the vacuolization of the nerve tissues. Accordingly, the rate of injuries in the pre- and post-treatment groups saw a significant decrease (400X, Scale bars=100 µm). Moreover, certain subcortical parts of the brain and the corpus callosum in the right hemisphere, which received middle cerebral artery perfusion, were stained using FLB (Figures 6 & 7).

On the other hand, the nerve tissues and fibers in this section were observed to be healthy in the control group (5), while in the ischemic-control group, destruction of nerve tissues, demyelination of nerve fibers and fragmentation of axons were visible in the nerve bundles under the cortex and in the corpus callosum (I-R). According to the findings of the present study, atorvastatin could significantly reduce the aforementioned injuries in the treated animals before and after the induction of ischemia. Consequently, the condition of nerve fibers and axons appeared to be normal in the treated animals, as well as in those in the control group (pre- and post-treatment).

On the other hand, the images provided from the studied animals in the control group (S) revealed the presence of healthy nerve fibers; however, a bundle of nerve tissue fibers was detected in the ischemic-reperfusion group, along with damaged myelin nerve fibers, and the level of this injury was observed to reduce significantly in the pre-treatment and post-treatment groups (400X, Scale bars=100 µm).

In addition, healthy fibers and nerve tissues were detected in the images of the control group, while in the ischemic-reperfusion control group, a bundle of nerve tissue fibers and damaged myelin nerve fibers were visible, which had a significant decrease in the pre- and post-treatment groups (400X, Scale bar=100 µm).

**Figure 6. Optical Microscopic Images from the Cross Section (under the Cortex) in the Right Hemisphere of Animals, 24 hours after Reperfusion (Stained via LFB method)**
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Figure 7. Optical Microscopic Images from Cross Sections of White Tissues (under the Cortex, Corpus callosum) in the Right Hemisphere of Animals, 24 hours after Reperfusion (Stained via LFB)

Discussion

According to the results of the present study, administration of atorvastatin before and after the induction of ischemia (i.e. at the onset of reperfusion) resulted in the significant reduction of lesion volumes in the ischemic hemispheres of rats, as well as the movement disorders. Furthermore, using this drug before the onset of ischemia and immediately after reperfusion could significantly reduce tissue swelling, as a key factor in the determination of brain edema. On the other hand, pathological investigations were indicative of a significant decrease in the lesion volumes of the nerve tissues, neurons and nerve fibers. Therefore, the findings of the current study confirm the protective effects of atorvastatin against strokes as to diminish the brain damage caused by ischemia-reperfusion. Ischemia and strokes lead to the activation of destructive factors and mechanisms in the brain, which could cause the death of neurons and other cells in the neuronal tissues (3).

Increased production of free radicals, activation of the inflammation system, release of excitatory neurotransmitter and programmed cell-death are among other factors caused by stroke (4). In the present study, induction of ischemia for 90 minutes was observed to cause lesions in the cortical and subcortical brain areas of the ischemic animals, which is considered as another prominent feature of strokes.

Brain tissues are easily affected by lipid peroxidation due to the presence of polyunsaturated fatty acids and weak antioxidant enzyme activity (11, 23). Therefore, different cells in brain tissues are prone to considerable damage and pathological changes during ischemia. In several studies conducted on the brain samples of animals with laboratory models of stroke, a variety of pathological changes have been reported; such examples are the formation of neuronal eosinophilia (i.e. acidophilic neurons with pyknotic, wrinkled and dense cores) and ghost neurons (i.e. less acidophilic neurons with darker cores than the natural form) (24,25).

In the current study, the aforementioned pathological changes, as well as neuronal tissue vacuolization, were detected in the brains of the studied animals 24 hours after the induction of ischemia. In addition, axon fragmentations and nerve tissue demyelination were observed in the corpus callosum, which is compatible with the findings of other studies regarding ischemic hemispheres.

According to different studies, other factors associated with pathological changes of stroke, which could damage nerve tissues and neurons, are as follows: 1) activation of inflammatory response (e.g. increased number of cytokines and proinflammatory chemokines), which could lead to the infiltration of the inflammatory cells (e.g. neutrophils) into the injured areas of the brain; 2) ionic imbalance; 3) cell swelling; 4) release of excitatory neurotransmitters and 5) toxicity caused by hyperstimulation (i.e. hyperexcitability) (3, 4, 26). Moreover, increased production of oxygen free radicals in brain ischemia, especially in the reperfusion stage, plays a key role in the damages caused by cerebral ischemia (27).

On the other hand, the combination of these radicals with free radicals of nitrogen (nitric oxide) may lead to the release of a dangerous compound called Peroxynitrite in the ischemic area of the brain, which is the most important contributor of tissue necrosis and induction of programmed cell-death (28). Accordingly, restoration of perfusion in the ischemic area, as well as the production and accumulation of destructive factors, could significantly reduce the consequences and mortality rate among stroke patients (1). Despite the remarkable advances in the comprehension of pathophysiological aspect of ischemic stroke, the treatment options are still limited.

Due to their beneficial properties including anti-inflammatory effects, protective effects against oxidative stress and inhibition of platelet aggregation in several pathological conditions, statins have compelled many researchers (11, 29, 30). In the present study, the use of atorvastatin in both treatment groups before ischemic initiation and at the onset of reperfusion, was
observed to significantly reduce the volume of brain lesions in the ischemic groups while improving movement disorders as well. In a study by Carloni et al., the use of simvastatin, another medicine in the statin family, was also reported to reduce the volume of the injuries caused by ischemia and right carotid artery occlusion, as well as hypoxia (31).

In another study in this regard, Mariucci et al. reported that the intravenous administration of pravastatin was able to reduce brain edema and lesion volume following the occlusion of the middle cerebral artery (32); it is also noteworthy that pravastatin belongs to the family of statins.

Similarly, the findings of the current study indicated that the administration of atorvastatin was able to decrease the swelling of brain tissues, as an index in the evaluation of edema, before and after ischemic-reperfusion in the brain of rats. In the present study, atorvastatin was found to prevent the demyelination of nerve fibers, as well as the fragmentation of available axons in the corpus callosum. Moreover, it was observed to arrest the vacuolization of nerve fibers and decrease the number of eosinophilia neurons after the ischemic-reperfusion. Based on the argument that the increased production of oxygen free radicals and inflammatory factors are among the major risk factors of stroke and ischemia, it could be concluded that in the current study, atorvastatin treatment was able to reduce brain lesions through the inhibition of the aforementioned directions. The anti-oxidative and anti-inflammatory effects of this medicine have been well documented by several studies (30,33).

Many researchers have attributed these properties to the inhibited production of Mevalonic acid, which is in the pathway of isoprenoid synthesis. Mevalonic acid is a production of the HMG-CoA reductase enzyme, and it is the origin of many isoprenoids, which, apart from cholesterol synthesis, play a pivotal role in intracellular processes such as apoptosis, inflammation, coagulation, and leukocyte adhesion and transmigration (34, 35). Accordingly, the inhibition of isoprenoid synthesis by statins could develop a defensive mechanism during the harmful cellular events activated at the time of stroke.

In a study by Hong et al., the subcutaneous injection of atorvastatin was observed to prevent the production of superoxide anion through reducing the activity of NADPH oxidase (36). This enzyme is the dominant source of reactive oxygen species in the central nervous system.

Another study by Wagner in this regard indicated that by increasing the activity of the endothelial enzyme of nitric oxide synthases and improving the cerebrovascular performance, atorvastatin could increase perfusion to the ischemic area of the brain, preventing platelet aggregation and adhesion (37).

In conclusion, the findings of the present study indicated that atorvastatin could significantly reduce the damages caused by ischemic-reperfusion injuries, while improving motor neuron disorders after stroke. Furthermore, as a neuroprotective agent, atorvastatin was capable of preventing neuron destruction in the ischemic areas of the brain. Therefore, this drug could be effectively used in the prevention and diminishing the side effects of strokes independently, or in combination with other ischemia medications.

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