

Effect of immune modulation on brain ischemia reperfusion injury

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Abstract

Cerebral ischemia–reperfusion injury is a complex process resulting in cellular damage and death. Ischemia and reperfusion in the brain induces an inflammatory response which may exacerbate initial levels of tissue injury. In this study, we investigated the possible immune modulation of rosuvastatin in brain ischemia reperfusion injury via interfering with inflammation. Twenty four adult albino rats were randomized into four groups (each of 6) as follow: Group (1) sham group: the rats were subjected to the same surgical procedures as other groups but the common carotid arteries were not occluded; Group (2) control (ischemic-reperfused) group: the rats were subjected to the same surgical procedures as other groups with bilateral common carotid arteries occlusion (BCCAO) for 30 min. followed by reperfusion for 1 hr but without drug; Group (3) control vehicle group: three days before surgery, rats received daily the vehicle of rosuvastatin drug, normal saline (0.9% NaCl) (1 ml/kg/day) intraperitoneally, then anesthesia and surgery with BCCAO for 30 min. followed by reperfusion for 1 hr were done and Group(4) rosuvastatin treated group: rats received daily rosuvastatin intraperitoneally. The dose of rosuvastatin was (10 mg/kg /day) for three days before the surgery, then anesthesia and surgery with BCCAO for 30 min. followed by reperfusion for 1 hr. At the end of the experiment, the levels of cerebral IL-6 significantly ($p < 0.05$) increased in control group as compared with the sham group. Histopathological analysis showed that rats in control group showed significant cerebral injury. Treatment with rosuvastatin significantly counteracted the increase in the cerebral levels of IL-6. Histopathological analysis revealed that rosuvastatin significantly reduced the severity of cerebral injury in the rats underwent BCCAO. We concluded that inflammatory cytokines are involved in global cerebral ischemia induced by bilateral common carotid arteries occlusion. Cerebral ischemia reperfusion injury can be modified by rosuvastatin via its anti-inflammatory effect. The aim of the study is to investigate the possible immune modulation of rosuvastatin in brain ischemia reperfusion injury.

Keywords: Cerebral ischemia reperfusion injury, inflammation, rosuvastatin

تأثير التحوير المناعي على الإصابة بنقص التروية الدماغية وإعادة ضخه
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الخلاصة:

ان الإصابة بنقص التروية الدماغية وإعادة ضخه هي من العمليات المعقدة التي تؤدي إلى تلف الخلايا ثم موتها كما ان نقص التروية الدماغية وإعادة الضخ، يحفز استجابة التهابية والتي بدورها قد تؤدي إلى تقاوم المستويات الأولية لإصابات الأنسجة. في هذه الدراسة فحصنا امكانية التحويل المناعي للرزوفاستاتين في الاصابة بنقص التروية الدماغية وإعادة ضخه من خلال التداخل مع الالتهاب. الهدف من الدراسة هو البحث عن امكانية التحويل المناعي للرزوفاستاتين عند الاصابة بنقص التروية الدماغية وإعادة ضخه. في هذه الدراسة، تم استعمال 24 ذكر من ذكور الجرذان البيضاء البالغة وتم تقسيمها عشوائيا إلى اربعة مجاميع (6 في كل مجموعة) كالآتي:

1- مجموعة التظاهر: الجرذان تعرضت لنفس العملية الجراحية كما في المجاميع الأخرى لكن الشرايين السباتية المشتركة لم تكن مسدودة.

2- مجموعة السيطرة: الجرذان تعرضت لنفس العملية الجراحية كما في المجاميع الأخرى مع انسداد ثنائي للشرايين السباتية المشتركة الى 30 دقيقة يليها إعادة الضخ لساعة واحدة لكن دون عقار.

3- مجموعة الحامل: ثلاثة أيام قبل الجراحة، الجرذان تلقت يوميا حامل عقار الرزوفاستاتين، المحلول الملحي كلوريد الصوديوم 9% (1مل/كغم/يوم) , داخل الصفاق ثم تخدير وجراحة انسداد ثنائي للشرايين السباتية المشتركة الى 30 دقيقة يليها إعادة الضخ لساعة واحدة.

4- المجموعة التي تلقت علاج الرزوفاستاتين: الجرذان تلقت يوميا الرزوفاستاتين داخل الصفاق. جرعة الرزوفاستاتين كانت (10مل/كغم/يوم) الى ثلاثة أيام قبل الجراحة، ثم تخدير وجراحة انسداد ثنائي للشرايين السباتية المشتركة الى 30 دقيقة يليها إعادة الضخ لساعة واحدة.

في نهاية التجربة، المستوى الدماغي ل IL-6 زاد معنويا ($p < 0.05$) في مجموعة السيطرة مقارنة بمجموعة التظاهر. التحليل النسيجي اظهر بان الجرذان في مجموعة السيطرة أظهرت اصابه دماغية هامه. العلاج رزوفاستاتين كافح بشكل كبير الزيادة في المستوى الدماغي ل IL-6. التحليل النسيجي كشف بأنه رزوفاستاتين خفض بشكل كبير ($p < 0.05$) من شدة الاصابة الدماغية في الجرذان التي خضعت لانسداد ثنائي للشرايين السباتية المشتركة.

نتائج الدراسة اظهرت ان السايٲوكينات الالتهابية تشارك في نقص التروية الدماغية الشامل الناجم من انسداد ثنائي للشرايين السباتية المشتركة. الاصابة بنقص التروية الدماغية وإعادة الضخ يمكن تعديله بالرزوفاستاتين عبر تأثيره المضاد للالتهابات.

الكلمات الافتتاحية: الاصابة بنقص التروية الدماغية وإعادة ضخه، التهاب، رزوفاستاتين.

Introduction

The term ischemia reperfusion injury (IRI) describes the experimentally and clinically prevalent finding of tissue ischemia with inadequate oxygen supply followed by successful reperfusion initiates a wide and complex array of inflammatory responses that may both aggravate local injury as well as induce impairment of remote organ function¹. Ischemia-reperfusion injury contributes to the pathophysiology of many conditions, include the different forms of acute vascular occlusions such as stroke, myocardial infarction, peripheral vascular insufficiency and hypovolemic shock with the relevant reperfusion strategies like thrombolytic therapy, coronary angioplasty,

cardiopulmonary bypass and operative revascularization². Cerebral ischemia leads to energy depletion and cell death, which can stimulate immune responses, leading to inflammatory cells activation and infiltration. Reperfusion of the occluded vessel, either spontaneously or by the collateral circulation or by therapeutic recanalization, leads to the generation of reactive oxygen species (ROS) that are delivered with the reperfused oxygenated blood or produced within brain and immune cells. ROS can then stimulate ischemic cells, even ischemic neurons, to secrete inflammatory chemokines and cytokines that enhance the biosynthesis of adhesion molecule in the cerebral vasculature and also lead to peripheral leukocyte recruitment³. As inflammatory cells

become activated, they can release a variety of cytotoxic molecules including more cytokines, matrix metalloproteinases (MMPs), nitric oxide (NO) and more ROS. These molecules may provoke more cell damage as well as disruption of the extracellular matrix and blood-brain barrier (BBB) ^{4,5}. Secondary ischemic brain damage occurs as a consequence of brain edema and post-ischemic microvascular stasis leading to hypoperfusion and post-ischemic inflammation ^{6,7}. The recruitment of peripheral circulating leukocytes into the brain parenchyma can produce an augmentation of inflammatory signal cascades, which will enhance the damage. These processes are mainly prominent during reperfusion which is often associated with microvascular injury, particularly due to increased permeability of capillaries and arterioles that lead to an increase of fluid filtration across the tissues. These activated endothelial cells produce more ROS following reperfusion, and results in a subsequent inflammatory response. White blood cells, carried to the area by the newly returning blood, release a mass of inflammatory factors such as interleukins (ILs) as well as free radicals in response to tissue damage. The restored blood flow reintroduces oxygen within cells that damages cellular proteins, DNA, and the plasma membrane. Damage to the cell's membrane may in turn causes the release of more free radicals. ROS may also act indirectly in redox signaling to turn on apoptosis. White blood cells may also bind to the endothelium of small capillaries, obstructing them and leading to more ischemia ^{8,9}. Early restoration of blood flow remains the treatment of choice for limiting brain injury following ischemic stroke. Improved educational efforts that emphasize the early signs and symptoms of stroke, coupled with the widespread application of thrombolytic

therapy to patients with acute ischemic stroke have increased the number of patients benefiting from reperfusion ¹⁰. While reperfusion of the ischemic brain is desirable, tissue damage may result from reperfusion *only*. Reperfusion appears to enhance the inflammatory response and causes additional injury to adjacent brain tissue ¹¹. From experimental stroke, blocking various aspects of the inflammatory cascade has shown to improve injury ⁸. Rosuvastatin belongs to a new generation of statins which are 3-hydrox-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. The study of Ma et al, 2013 ¹² showed that pretreatment with rosuvastatin extensively protects against reperfusion injury after middle cerebral artery occlusion, as shown by inhibition of neuronal cell death, and these helpful effects were associated with suppression of oxidative stress or inflammation-related pathways, such as reduction of an increase in cerebral superoxide and NADPH oxidase subunits, suppression of activation of microglia and macrophage, and inhibition of upregulation of NF-KB, iNOS and COX-2, so pretreatment with rosuvastatin reduced neuronal cell apoptosis in reperfusion injury through inhibition of inflammation ¹².

Materials and methods

Animals

The study was performed using twenty four adult albino rats weighting (200-250 g), provided by the animal house of high institute of infertility diagnosis and assisted reproductive technologies / Al-Nahrain university. The rats were housed in the animal house of faculty of pharmacy/ Kufa University, in a room in which lighting was controlled (12 hr on, 12 hr off), temperature was kept at (25±1°C) and humidity was kept at (60–65%) with unlimited access to food and water until the start of experiments. The Animal Investigation Committee(AIC) office of Kufa university approved the experimental protocol.

Preparation of rosuvastatin

Rosuvastatin was supplied by (Pioneer co. Sulaymaniyah/Kurdistan Iraq), and was prepared immediately before use by dissolving it in normal saline.

Experimental groups

After one week of acclimatization, the rats were divided randomly into four equal groups (each of 6) as follow: Group (1) sham group: The rats were subjected to the same surgical procedures as other groups but the common carotid arteries were not occluded ; Group (2) control (ischemic-reperfused) group: The rats were subjected to the same surgical procedures as other groups with bilateral common carotid arteries occlusion (BCCAO) for 30 min. followed by reperfusion for 1 hr but without drug; Group (3) control vehicle group: three days before surgery , rats received daily the vehicle of rosuvastatin drug, normal saline (0.9% NaCl) (1 ml/kgB.W/day), intraperitoneally (iP) ^{13,14} then anesthesia and surgery with BCCAO for 30 min. followed by reperfusion for 1 hr were done; Group (4) rosuvastatin treated group: the rats received daily rosuvastatin intraperitoneally (iP). The dose of rosuvastatin was (10 mg/kgB.W /day) for three days before the surgery ^{13,14}, then anesthesia and surgery with BCCAO for 30 min. followed by reperfusion for 1 hr were done.

Induction of global brain ischemia

Each rat was anesthetized by intraperitoneal (iP) injection of 100 mg/kg of ketamine and 5 mg/kgB.W of xylazine ¹⁵. Within few min, the rat became unconscious, then placed in supine position and exposed to light source to keep it worm. After that a midline ventral small skin incision in the neck was made and the paratracheal muscles and fascia were splitted and pulled by stay sutures to expose the trachea, carotid arteries and vagal nerves. Both common carotid arteries were exposed, with special attention paid to separate and preserve the vagus nerve fibers and global cerebral ischemia was induced by BCCAO ¹⁶ by using vascular clamps for 30 min. After 30 min of global cerebral ischemia, the clamps were removed to allow the reflow of blood through carotid arteries (reperfusion) for 1 hr ¹⁷.

Preparation of samples**Tissue preparation for IL-6 measurement**

After decapitation, the brain was removed and washed in cold normal saline (0.9% NaCl) to remove any blood or debris and subsequently blotted on filter paper. Afterward, brain tissues were homogenized in ice-cold 1:10 (w/v) 0.1 M phosphate-buffered saline (PBS) (pH 7.4), containing 1× protease inhibitor cocktail and 0.2% Triton X-100 for 30 seconds, using a high intensity ultrasonic liquid processor. The resulting homogenates were centrifuged at 15,000 g for 30 min, at 4°C, and supernatants were stored at -80°C until analysis was done ¹⁸. IL-6 was measured by ELISA test by using rat IL-6 ELISA kit which was supplied by BIOTANG Inc, USA.

Tissue preparation for histopathological analysis and Scoring of brain damage

After 30 min. ischemia and 60 min. reperfusion, decapitation was done and the brain was removed and fixed with 10% formalin and embedded in paraffin wax and cut into coronal sections of 4-8µm thickness. The sections were stained with haematoxylin and eosin (H&E) dye for histopathological examination that was done by pathologist. The scoring system for the pathological changes in ischemia reperfusion injury is as follows ¹⁹ : 0:(normal) = no morphological signs of damage; 1:(slight) = edema or eosinophilic or dark neurons (pyknotic) or dark/ shrunk cerebellar Purkinje cells; 2:(moderate)= at least two small hemorrhages; 3:(severe) = clearly infarctive foci (local necrosis).

Statistical analysis

All data are expressed as mean ± SE. The difference between various groups were analyzed by one-way analysis of variance (ANOVA) followed by multiple comparison tests as Post Hoc. LSD. Non-parametric tests were used to assess the statistical significance of histopathological parameter. Cerebral lesions is a non-normally distributed variable. The Fisher exact test is used when members of two independent groups can fall into one of two mutually exclusive categories. The test is used to determine whether the proportions of those falling into each category differ by group. In all tests $P < 0.05$ was considered to be statistically significant.

Results

Effect on inflammatory marker (IL-6)

At the end of the experiment, the level of cerebral IL-6 significantly ($P < 0.05$) increased in control group as compared with sham group. The level of cerebral IL-6 of rosuvastatin treated group were

significantly ($p < 0.05$) lower than that of control and control-vehicle group. The values of cerebral IL-6 are shown in figure (1).

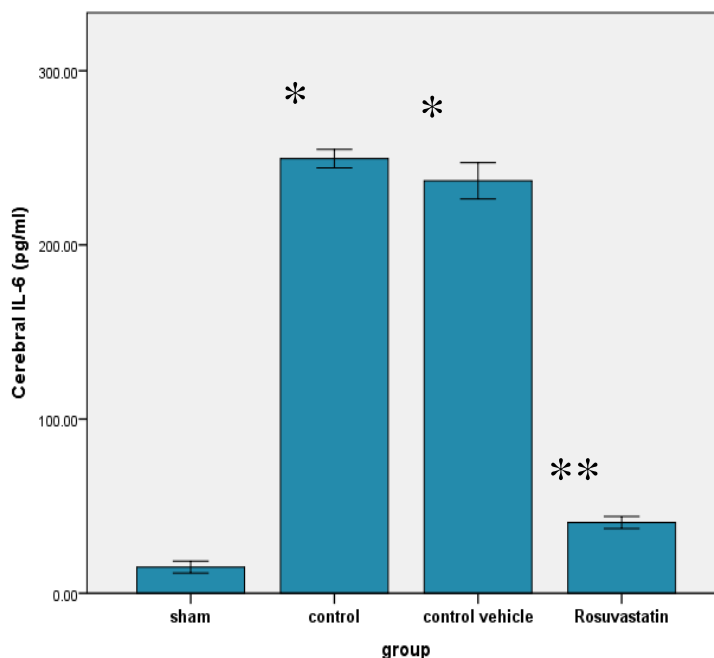


Figure (1): Error bar chart shows the difference in mean \pm SEM values of cerebral IL-6 level (pg/mg) in the four experimental groups at the end of the experiment (No. of animals = 6 in each group). * $P < 0.05$ vs. sham group, ** $P < 0.05$ vs. control-vehicle group.

Histopathological findings

The cerebral injury was assessed in the rat's brain of the four experimental groups according to ¹⁹ and the results were as follow: In the sham group, a cross sections of rat's brain showed normal appearance (100%) as shown in table (1) and figure (2). Statistically, there was a significant difference between control group and sham group, and the score of the control group showed that (66.6%) of the group had severe cerebral injury, (16.7%) had moderate cerebral injury and (16.7%) had slight cerebral injury as shown in table (1) and figure (2). Statistically, there was insignificant difference between control group and vehicle control group, and the score of the control vehicle group showed that (16.7%) had severe cerebral injury, (66.6%) had moderate cerebral injury and (16.7%) had slight cerebral injury as shown in table (1) and figure (2). pretreatment of rats with rosuvastatin improved cerebral injury score significantly as compared with control vehicle group and the score of this group showed that (16.7%) had normal histopathological appearance, (50%) had slight cerebral injury and (33.3%) had moderate injury as shown in table (1) and figure (2). The histopathological cerebral changes are shown in figures (3-8).

Table (1): The differences in histopathological grading of cerebral changes among the four experimental groups.

Histopathological Score	Groups								
	Sham		Control		Control vehicle		Rosuvastatin		
	N	%	N	%	N	%	N	%	

Score 0(normal)	6	100	0	0	0	0	1	16.7
Score 1(slight)	0	0	1	16.7	1	16.7	3	50
Score2(moderate)	0	0	1	16.7	4	66.6	2	33.3
Score 3(sever)	0	0	4	66.6	1	16.7	0	0
Total	6	100	6	100	6	100	6	100

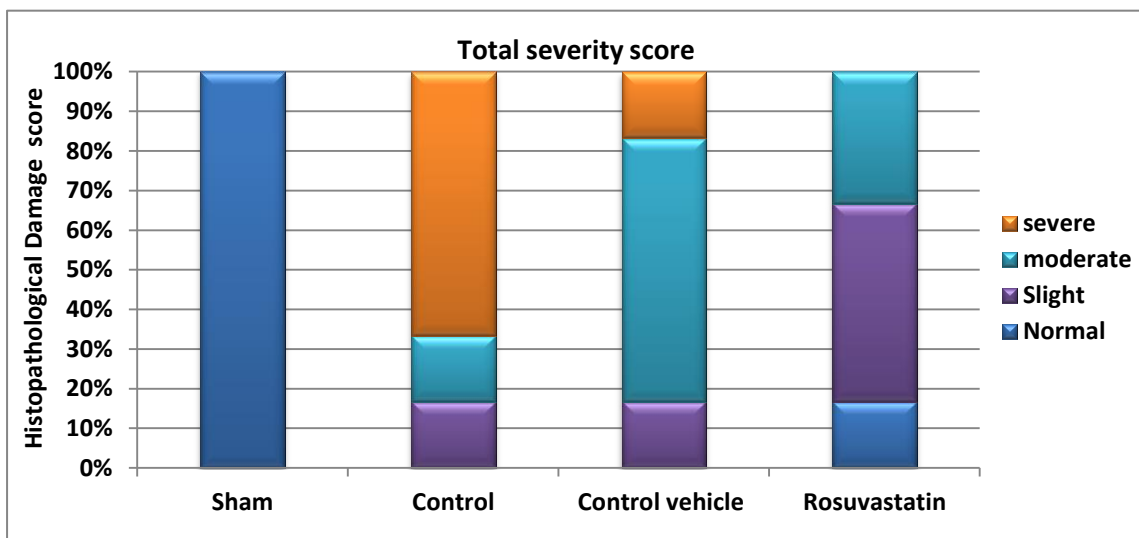


Figure (2): Component bar chart shows the relative frequency of different histopathological grading of cerebral changes among the four experimental groups.

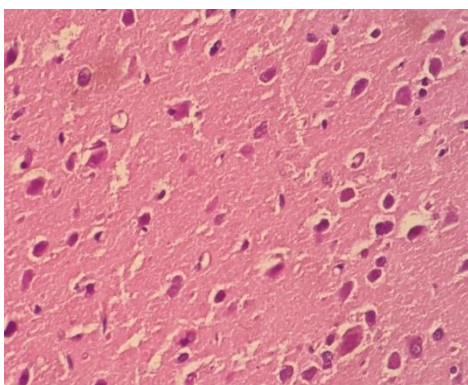
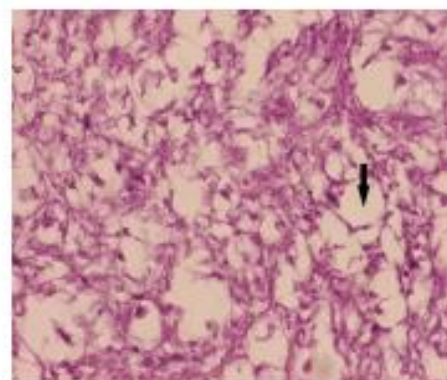


Figure (3): A Photomicrograph of normal rat's brain section shows normal tissue and the histopathological score =0. The section stained with H&E (X 40).



Figure(4): Photomicrograph of rat's brain section after global cerebral ischemia shows edema (black arrow) and the histopathological score = 1 (Slight injury). The section stained with H&E (X 40).

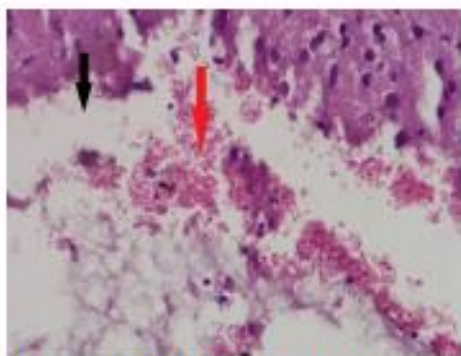


Figure (5): Photomicrograph of rat's brain section after global cerebral ischemia shows edema (black arrow)

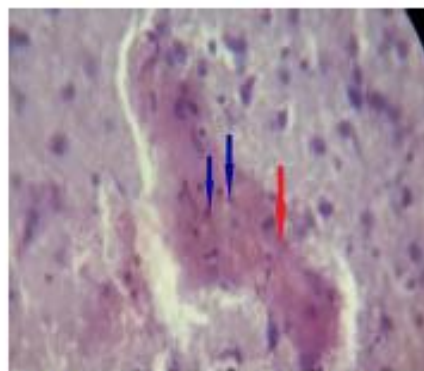


Figure (6): Photomicrograph of rat's brain section after global cerebral ischemia shows edema (black arrow) and neutrophil infiltration (blue arrow). The histopathological score = 2 (Moderate injury). The section stained with H&E (X 40).

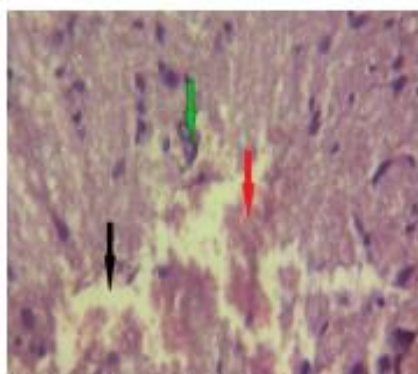


Figure (7): Photomicrograph of rat's brain section after global cerebral ischemia shows edema (black arrow), hemorrhage (red arrow) and area of necrosis and destroyed neuron (green arrow). The histopathological score = 3 (Moderate injury). The section stained with H&E (X 40).

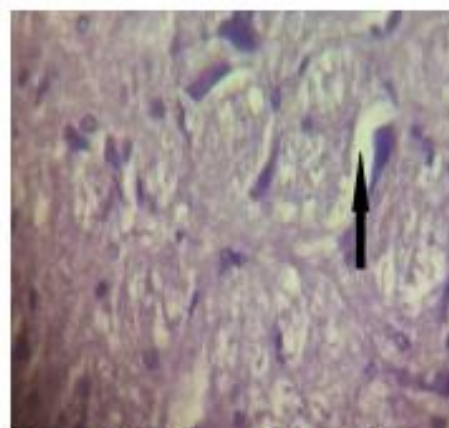


Figure (8) : Photomicrograph for brain tissue of rats treated with rosuvastatin drug shows mild edema (slight injury). The histopathological score =1 . The section stained with H&E (X 40).

Discussion

Effect of global cerebral ischemia reperfusion injury on IL-6

In the present study, a significant increase ($P < 0.05$) in IL-6 level was found in the

control group as compared with the sham group. **Chu et al. (2012)**²⁰ showed that transient global cerebral ischemia reperfusion injury resulted in a substantial increase in the mRNA expression levels of TNF- α and IL-6

in the rat hippocampus. Jing et al. (2012)²¹ data indicate that inflammatory response was initiated after transient cerebral ischemia and the release of inflammatory cytokines such as IL-6 and TNF- α occurred in the brain. Higher IL-6 levels have been detected in the peripheral blood of patients with acute cerebral ischemia than in control subjects^{22,23}. Increased plasma and cerebrospinal fluid IL-6 levels are correlated with a larger infarct size²².

Effect of global cerebral ischemia reperfusion injury on cerebral histopathology

There was a statistically significant difference between control group and sham group. The score of the control group shows slight and moderate cerebral injury. From the histopathological study of Prakash et al.(2011)²⁴, it was observed that sections of brain tissue showed swollen neurons, dilated blood vessels with neuronal loss occurred in brain regions of ischemic reperfused rats induced by BCCAO for 30 min followed by 1 hr and 4 hr reperfusion in ischemic control group. While no apparent morphological changes in sham and brain section showing normal structure. Chandrashekar et al.(2010)¹⁷ demonstrated that global cerebral ischemia on rats by BCCAO for 30 min followed by 1 hr reperfusion caused marked congestion of blood vessels and neutrophil infiltration and neuronal necrosis. Shah et al. (2005)²⁵ found that in BCCAO for 30 min, caused marked congestion of blood vessels and these effects were further augmented following reperfusion for 1hr i.e. lymphocytic proliferation and neuronal necrosis.

Effect of rosuvastatin on IL-6

The present study showed that rosuvastatin administration before the induction of cerebral ischemia caused a significant lowering ($P < 0.05$) in cerebral level of IL-6 as compared with control and control vehicle groups. So our results indicated that rosuvastatin can prevent cerebral inflammation and decrease ischemic brain damage. This finding is in agreement with Sironi et al.(2005)²⁶ who found that rosuvastatin attenuates inflammatory processes associated with cerebrovascular disease. Awad and El Sharif.(2010)²⁷ showed that rosuvastatin pretreatment appeared to protect the liver, lung, kidney, intestine, and heart tissues after hepatic ischemia reperfusion

injury through the reduction of proinflammatory cytokines (TNF- α , IL-6, and MCP-1) and stimulation of anti-inflammatory cytokines (IL-10) production. So this data suggested a therapeutic potential for rosuvastatin in attenuating inflammation and modulating immune response independent of lipid lowering effect. Li et al.(2005)²⁸ demonstrated that treatment with rosuvastatin has acute anti-inflammatory actions that likely participate in its beneficial actions during atherogenesis. Liu et al.(2014)²⁹ demonstrated that in hypertensive patients with carotid atherosclerosis, there was a significant effect of rosuvastatin on reducing carotid intima-media thickness (IMT), IL-17, IL-6, IL-23 and TNF- α .

Effect of rosuvastatin on brain histopathology

In the current study, pretreatment with rosuvastatin for 3 days before cerebral ischemia, ameliorated the brain injury significantly as compared with control group. Xing et al.(2006)³⁰ demonstrated that rosuvastatin could remarkably decrease infarct volume and cerebral edema after MCAO. This effect could be attributed to the pleotropic effect of rosuvastatin as anti-inflammatory, anti-oxidant and anti-apoptotic agent.

Acknowledgement

We are grateful for everyone help us in our work.

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