Effects of sodium β-aescin on expression of adhesion molecules and migration of neutrophils after middle cerebral artery occlusion in rats

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KEY WORDS brain ischemia; aescin; neutrophils; peroxides; intercellular adhesion molecule-1; E-selectin

ABSTRACT

AIM: To investigate the effects of sodium β-aescin on neutrophil migration and expression of adhesion molecules (ICAM-1 and E-selectin) after middle cerebral artery occlusion (MCAO) in rats. **METHODS:** Rats were pretreated with sodium β-aescin for 7 d and then subjected to cerebral ischemia/reperfusion (I/R) injury induced by an MCAO. After a 2-h ischemia and a 24-h reperfusion, the infarct volume and neurological deficit were determined by the method of TTC staining and the Longa's score. The effect of sodium β-aescin on the migration of neutrophils was evaluated by measuring the activity of myeloperoxidase (MPO) enzyme. The expressions of adhesion molecules were determined by immunohistochemistry and Western blot. **RESULTS:** Sodium β-aescin significantly reduced the cerebral infarct volume and ameliorated the neurological deficit ($P<0.05$ or $P<0.01$). The MPO activity and the expressions of ICAM-1 and E-selectin in the vehicle-treated rats were increased significantly ($P<0.01$) after cerebral I/R. After treatment with sodium β-aescin, the enzymatic activity of MPO and the expressions of these adhesion molecules were significantly reduced compared with the vehicle-treated group ($P<0.05$ or $P<0.01$). **CONCLUSION:** Sodium β-aescin can attenuate brain injury, down-regulate the protein expressions of ICAM-1 and E-selectin, and reduce the migration of neutrophils after cerebral I/R.

INTRODUCTION

An inflammatory reaction is a common response of the brain parenchyma to various forms of insult. It is characterized by the infiltration of leukocytes which in the case of cerebral ischemia are mainly polymorphonuclear leukocytes and monocytes/macrophages[1]. An increased number of polymorphonuclear leukocytes in the microvessels or ischemic brain parenchyma have been found in various species[2,3]. Neutrophils (PMNs) are critically involved in the earliest stages of inflammatory reaction after tissue ischemia, initiating scavenger functions which are later subsumed by macrophages. However, there is dark side to neutrophil influx, especially in postischemic tissues, where activated neutrophils may augment damage to vascular and parenchymal cellular elements[4-7]. Experimental evidence points to a pivotal role for endothelial cells in establishing postischemic PMNs recruitment, in that hypoxic/ischemic endothelial cells synthesize the proinflammatory cytokines, such as IL-1, IL-8, and TNF-α[8,9]. These cytokines are considered to be among the principal

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mediators of immunologic and inflammatory responses, which can attract leukocytes and stimulate the synthesis of adhesion molecules in leukocytes, endothelial cells, *etc*, thus promoting the inflammatory responses of damaged cerebral tissue[10,11]. The expression of these intercellular adhesion molecules (such as ICAM-1 and E-selectin) is induced by various inflammatory process, such as cerebral ischemia, and upregulated by many cytokines[12-16].

β-Aescin is the major active principle from *Aesculus hippocastanum (Hippocastanace*ae), the horse chestnut tree. β-aescin is widely employed in the clinical therapy of vascular disorders and can attenuate neuronal damage induced by cerebral injury because of its anti-inflammatory, anti-oedematous, anti-edema and anti-oxidation effects[17]. However, the mechanism of the protective effects of sodium β-aescin after brain injury induced by cerebral I/R was unclear. The study was to investigate the effects of sodium β-aescin on neutrophil migration and expression of adhesion molecules after middle cerebral artery occlusion in rats.

MATERIALS AND METHODS

Drugs and reagents Sodium β-aescin (purity >98.5 %) was kindly supplied by Wuhan Aimin Pharmaceutical Factory. It was dissolved in saline before use; nimodipine (Nim) injection was from Bayer Company(Germany). All other chemicals and solvents were of analytical grade.

Animal treatment and administration Male Sprague-Dawley rats (Grade II, Certificate No 19-050) weighing 230-280 g were obtained from the Experimental Animal Center of Tongji Medical College, which were fed at a constant room temperature of 22 ºC under a 12-h light-dark cycle. Animals were treated with vehicle (0.9 % NaCl solution), sodium β-aescin (15, 30, and 60 mg/kg) dissolved in 0.9 % NaCl solution for 7 d, and nimodipine (0.7 mg/kg) respectively by ip injection 1 h before middle cerebral artery occlusion (MCAO). Six treatment groups were studied: 1) Sham-operated; 2) Vehicle-treated I/R (Model group); 3), 4), and 5) Sodium β-aescin-treated I/R (15, 30, and 60 mg/kg), 6)nimodipine-treated I/R.

Cerebral ischemia/reperfusion (I/R) procedure Rats were anesthetized with 10 % chloral hydrate (350 mg/kg, ip). The brain I/R injury was induced by a MCAO[18]. The right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) were isolated via a ventral midline incision. A 50-mm length of monofilament nylon suture (ϕ 0.22-0.24 mm), with its tip rounded by heating near a flame, was introduced into ECA lumen and advanced into the ICA for a distance of 18-20 mm in order to block the origin of the MCA. The body temperature of the rats was maintained at 37±0.5 ºC during the surgical procedure with an infrared heat lamp. Sham-operated animals were not exposed to I/R. After 2 h of ischemia, the nylon suture was withdrawn (reperfusion). After arousal from anesthesia, the rats were returned to the cages. All experimental procedures handling animals were conducted according to our institutional guideline for animal care.

Behavioral testing and determination of the area of cerebral damage After 2 h-ischemia and 24 h-reperfusion, neurological findings were scored on a five point score according to the Longa's method[18], and then the brains were removed and five coronal sections (2 mm thick slices from anterior 5.5 mm to anterior 13.5 mm) were preselected using a brain matrix. Sections were immersed in 2 % triphenyltetrazolium chloride (Sigma) in normal saline and incubated at 37 ºC for 15 min, and were fixed with 10 % formalin neutral buffer solution (pH 7.4). Infarct brain was identified as an area of unstained tissue. Infarct volumes were calculated from planimetic analysis of digitized imagines of serial cerebral sections and expressed as the percentage of infarction in ipsilateral hemisphere[19,20].

Measurement of PMNs infiltration The enzymatic activity of MPO was measured as an indicator of tissue of PMNs migration[21]. After I/R or sham operation, the brains were removed. The 100 mg of tissue (cortex and caudate putamen) was isolated and stored at -70 ºC for later biochemical analysis. The method of assay MPO activity was according to the guide of the assay kit (Nanjing Jiancheng Bioengineering Institute, China).

Immunohistochemistry of the ICAM-1 and E-selectin After I/R or sham-operation , the rats were anesthetized with 10 % chloral hydrate (350 mg/kg ip). Then the rats were perfused with 200 mL 0.9 % NaCl solution and subsequently with 4 % paraformaldehyde in 0.1 mol/L phosphate buffer at pH 7.4. Brains were removed and post-fixed for 24 h in the same fixative. The post-fixed brains were cryoprotected in 25 % sucrose in PBS and the coronal sections (20 μm thick) were placed on a cryostat (LEICA CM1850, Germany). The sections between 3 and 4 mm posterior of the bregma were used for this study. Briefly, an ABC kit

(Zhongshan Biotechnology Company, Beijing, China) was used to localize the primary antibody. The diaminobenzidine (DAB) kit (Zhongshan Biotechnology Company, Beijing, China) was used to visualize the catalyzed peroxidase-reaction product. The mouse monoclonal antibody against ICAM-1(Santa Cruz Biotechnology) was used at a 1:500 dilution and the goat polyclonal antibody against E-selectin (Santa Cruz Biotechnology, also used in Western blot analysis) at a 1:200. At the same time, the negative controls were performed, which were incubated without the primary antibodies.

Analysis of the expressions of ICAM-1 and E-selectin (Western blot analysis) The rats were decapitated and the brains were removed after ischemia/reperfusion or sham operation. The brains were divided into ipsilateral (infarct) and contralateral (non-infarct) hemispheres. The ipsilateral tissue was used for the assay of the protein expression of ICAM-1 and E-selectin, and one side tissue of the sham-operated rats was used as control. Three volumes of PBS were added to the tissues and homogenized, then stop buffer was added to the mixture. The samples were lysated by sonication (180 W) for 6 min, and centrifuged at 5000×*g* for 10 min. And then 0.04×volume β-ME was added to the solution followed by 10-min boiling. Protein 5 μg per lane were loaded on 8 % SDS-polyacrylamid mini gels. After electrophoresis, proteins were electrically transferred to nitrocellulose membranes. The membranes were incubated in 5 % milk TBST at 4 ºC overnight and subsequently incubated with the primary antibodies at room temperature. The primary antibodies were either monoclonal rabbit antibodies against ICAM-1(PTG Lab, USA, 1:500 dilution) or goat polyclonal antibodies against E-selectin (Santa Cruz Biotechnology, 1:100 dilution). After being washed with TBST, the membranes were incubated with secondary antibodies (horseradish peroxidase-conjugated goat-anti-rabbit IgG) at room temperature for 50 min. The immunoblots were visualized using enhanced chemiluminescence detection reagents. The absorbance (*A*) of the band was measured using Kodak ID Imagine software. Data were evaluated as % of absorbance levels of the sham-operated group. The β-actin analysis was also performed[22].

Statistics Data were expressed as mean±SD and analyzed by *t*-test. For quantitative analysis of ICAM-1 and E-selectin with histoimmunochemistry method, each slice was observed under the light microscope (×200) and analyzed by computerized imagine analysis system (HPIAS-1000, Champion Imagine). Four microscopic fields per region per section were analyzed. $P<0.05$ was considered to be significant.

RESULTS

Effects on the area of cerebral damage, edema and neurological score Compared with the sham-operated group, the area of cerebral infarct volume was increased significantly in the rats in the vehicle-treated group ($P<0.01$). After treatment with sodium β-aescin or nimodipine, the infarct volume was significantly reduced, and the neurological deficit was ameliorated ($P<0.05$ or $P<0.01$, Tab 1).

Tab 1. Effects of sodium β-aescin on the area of cerebral damage and neurological score after 2-h ischemia and 24-h reperfusion in rats. *n*=6. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* vehicle.

Group	Dose/ mg·kg^{-1}	Ratio of infarct area (w/w) %	Neurological score
Vehicle		28±4	2.4±1.1
Sodium β-aescin	15	20±7	1.5±1.3
	30	14±4^{b}	1.02±0.05
	60	9.0±2.1^{c}	0.87±0.12^{b}
Nimodipine	0.7	11±5^{c}	2.0±1.2

Effect of sodium β-aescin on PMNs infiltration In vehicle-treated rats, the enzymatic activity of MPO in cortex and caudate putamen in the ischemic hemisphere was higher than that in the sham-operated group ($P<0.01$). After treatment with sodium β-aescin or nimodipine, the enzymatic activity of MPO in the ischemic hemisphere was dropped as compared with the vehicle-operated group ($P<0.05$, Tab 2).

Effects of sodium β-aescin on the expressions of ICAM-1 and E-selectin The Previous studies have reported that the expressions of ICAM-1 and E-selectin obviously were increased by the inflammatory cytokines and reached peak from 12-24 h after cerebral ischemia[23]. So the effects of sodium β-aescin on the expressions of ICAM-1 and E-selectin were investigated at 24 h after MCAO.

With immunohistochemistry, the expressions of these molecules were obviously identified on the microvascular endothelial cells in the ischemic hemispheres

Tab 2. Effects of sodium β-aescin on the enzymatic activity of MPO after a 2-h ischemia and a 24-h reperfusion in rats. *n*=6. Mean±SD. $^{b}P<0.05$ *vs* vehicle. $^{f}P<0.01$ *vs* Sham.

Group	Dose/ mg·kg^{-1}	MPO activity/U·g^{-1} Cortex	Caudate putamen
Sham		0.18±0.04	0.14±0.03
Vehicle		0.39±0.09^{f}	0.35±0.08^{f}
Sodium β-aescin	15	0.30±0.08	0.33±0.07
	30	0.29±0.06^{b}	0.30±0.05
	60	0.26±0.05^{b}	0.24±0.06^{b}

after ischemia/reperfusion, but few vessel was immunopositive neither in the contralateral hemispheres nor in the sham-operated cerebral hemisphere. Compared with the sham-operated group, the number and intensity of ICAM-1-positive or E-selectin-positive vessels increased significantly in the ischemic hemisphere in the vehicle-operated groups ($P<0.01$). After treatment with sodium β-aescin (30, 60 mg/kg) or nimodipine, the expressions of ICAM-1 and E-selectin on the cerebral microvascular endothelium were down-regulated ($P<0.05$ or $P<0.01$, compared with the vehicle-operated groups) (Fig 1A to 1J, Tab 3).

Tab 3. Effects of sodium β-aescin on expressions of ICAM-1 and E-selectin as measured by immunohistochemistry after middle cerebral artery occlusion (2-h ischemia and 24-h reperfusion) in rats. –, no expression; +, weak expression; ++, prominent expression; +++, intense expression. *n*= 6.

Group	Dose/mg·kg^{-1}	Intensity of immunostaining ICAM-1	E-selectin
Sham		–	–
Vehicle		+++	+++
Sodium β-aescin	30	++	++
	60	+	+
Nimodipine	0.7	+	+

The expressions of these adhesion molecules were also analyzed by Western blot. In this study the antibodies recognized ICAM-1(M_r=103 kDa) and E-selectin (M_r=130 kDa) in the brain protein extracts, respectively. The brain tissue obtained from the sham-operated group showed low expression levels of ICAM-1 and E-selectin. After 2-h ischemia and 24-h reperfusion, the expressions of ICAM-1 and E-selectin remarkably increased in ischemia hemisphere in the vehicle-operated groups as compared with the sham-operated groups ($P< 0.01$). As a confirmation of immunohistochemistry analysis, the inhibitory effect of sodium β-aescin (30, 60 mg/kg) on the expressions of ICAM-1 and E-selectin was obviously observed ($P<0.05$ or $P<0.01$, compared with the vehicle-operated group) (Fig 2A to 2D).

DISCUSSION

It was reported that sodium β-aescin reduced cerebral infarct volume and cerebral edema induced by brain injury[24]. This study demonstrated that sodium β-aescin reduced the cerebral infarct volume and ameliorated the neurological deficit in the rats after cerebral I/R, which showed that sodium β-aescin could attenuate brain injury. The above results were consistent with the previous reports[17,24].

A large amount of studies demonstrated that acute PMNs recruitment to cerebral tissue had deleterious effects in the early reperfusion period. In order to investigate whether sodium β-aescin affected the PMNs infiltration in the pathogenesis of evolving cerebral damage, and whether the effect contributed to the protective effect of sodium β-aescin on cerebral I/R injury, we used the murine model of transient focal cerebral ischemia consisting of intraluminal MCAO for 2 h followed by 24 h of reperfusion. The assay of MPO enzymatic activity showed that sodium β-aescin could inhibit the PMNs recruitment to cerebral tissue. However, there was considerable evidence that in the brain, an early PMNs influx was mediated by a variety of adhesion molecules, among which the most studied adhesion molecules were ICAM-1 and E-selectin. ICAM-1 was located on the endothelial surface, and its leukocyte counterpart, integrin CD11/CD18. E-selectin bound to an overlapping set of carbohydrate structures at leukocyte surfaces. A number of animal studies documented the upregulation of adhesion molecules correlated to the PMNs infiltration to the cerebral tissue[25]. So the methods of immunohistochemistry and Western blot were used to investigate the effects of sodium β-aescin on the expressions of ICAM-1 and E-selectin proteins. The results showed the weakly detectable expressions of ICAM-1 and E-selectin on the cerebral vessel endothelial cells in rats, but after 2 h of ischemia and 24 h of reperfusion , the expressions of ICAM-1 and E-selectin

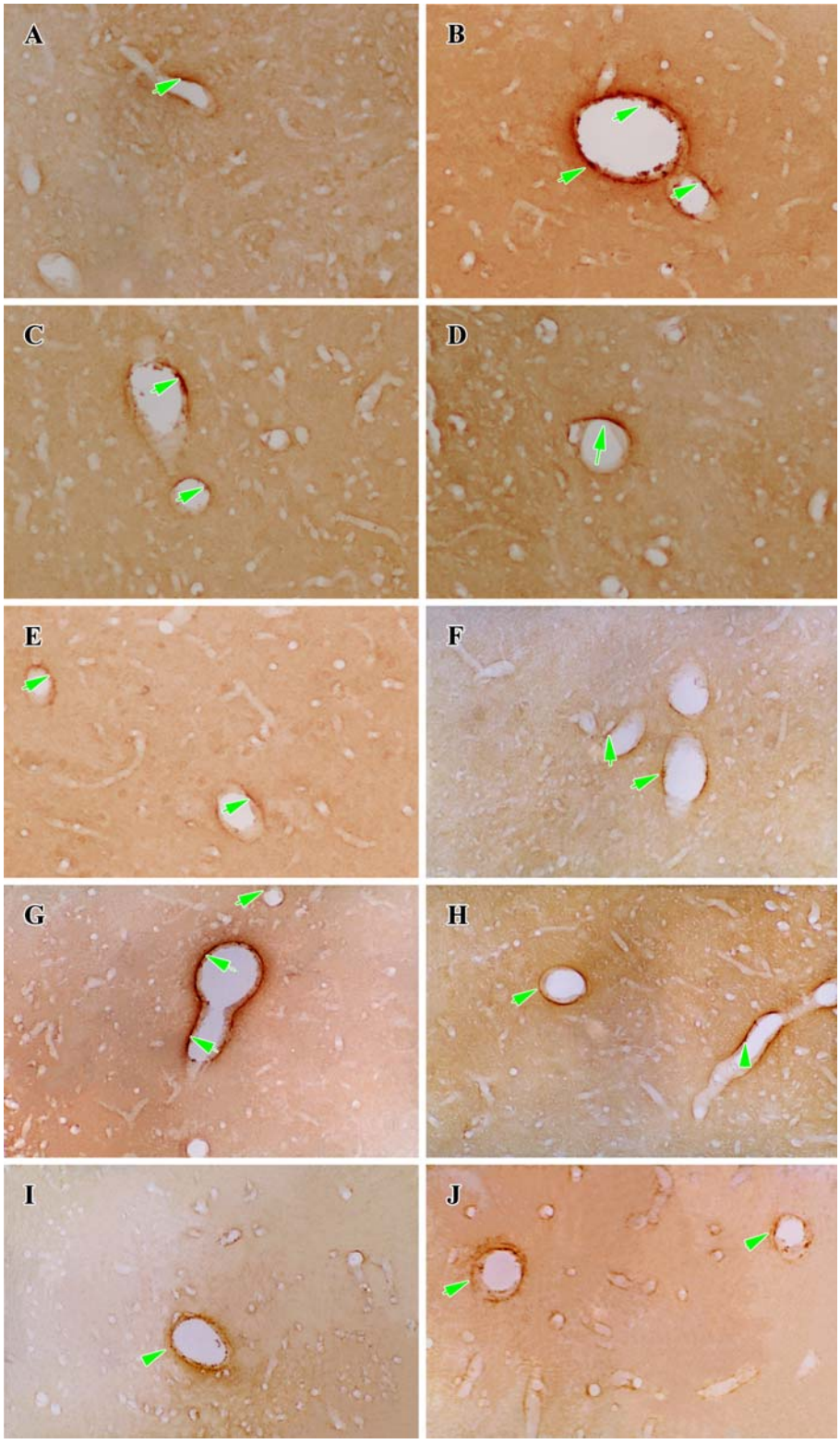

Fig 1. Representative photomicrographs of the expressions of ICAM-1 (A-E) and E-selectin (F-J) in coronal cerebral sections between 3 and 4 mm posterior of the bregma after a 2-h ischemia and a 24-h reperfusion in rats treated with or without β-aescin. (A, F): sham; (B, G): vehicle; (C, H): sodium β-aescin (30 mg/kg); (D, I): sodium β-aescin (60 mg/kg); (E, J): Nimodipine. Arrows indicate both ICAM-1- or E-selectin-positive cells. *n*=6. ×200.

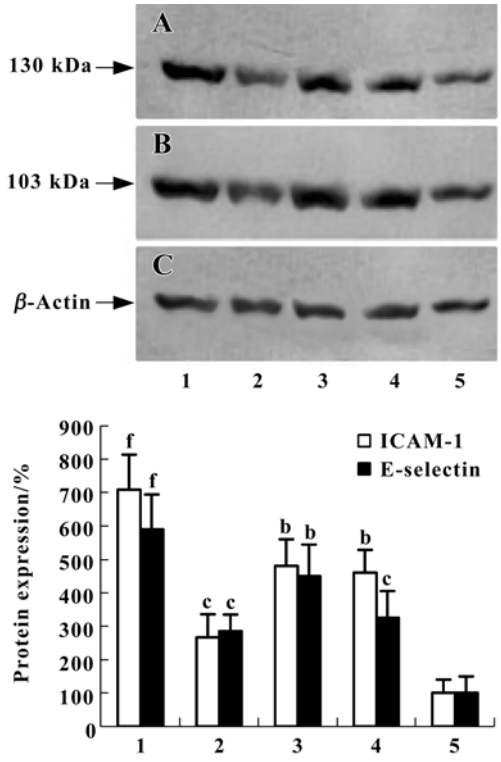

Fig 2. Western blot analysis of ICAM-1(A) and E-selectin (B) after middle cerebral artery occlusion (2 h of ischemia and 24 h of reperfusion) with β-actin protein as an internal control (C). The bands of ICAM-1 and E-selectin were located at 103 kDa and 130 kDa, respectively. Lane 1: vehicle; Lane 2, 3, and 4: sodium β-aescin (60, 15, and 30 mg/kg); Lane 5: sham. (D) Quantitative analysis of relative abundance of ICAM-1 and E-selectin immunoreactivity determined by densitometric measurement. The levels of ICAM-1 and E-selectin protein were expressed as % of absorbance levels of the vehicle group. *n*=5. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* vehicle. $^{f}P<0.01$ *vs* sham.

were increased significantly determined by either immunohistochemistry or Western blot. After treatment with sodium β-aescin, the inhibitory effect on upregluation of these adhesion molecules in rats were obviously observed, especially in sodium β-aescin groups of 30 and 60 mg/kg. In the light of the above consideration, sodium β-aescin can attenuate brain injury induced by cerebral ischemia/reperfusion partially through inhibiting inflammatory response, especially reducing the expressions of adhesion molecules and migration of neutrophil.

In conclusion, sodium β-aescin has the effects of inhibiting the activity of MPO enzyme and reducing the expressions of ICAM-1 and E-selectin on the cerebral vessel endothelial cells after cerebral ischemia/reperfusion. So we can draw the conclusion that sodium β-aescin has potential protective effects on brain injury after MCAO.

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