Inhibitory effect of β-aescin on inflammatory process following focal cerebral ischemia-reperfusion in rats

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Abstract: **AIM** To investigate if the beneficial effects of β-aescin on ischemia/reperfusion (I/R) induced cerebral injury are related to the inhibition of expressions of pro-inflammatory cytokines. **METHODS** Rats were pretreated ig with β-aescin for 7 d and then subjected to cerebral I/R injury induced by a middle cerebral artery occlusion. The infarct volume and the neurological deficit were determined by the method of TTC staining and the Longa's score. The permeability of the blood-brain barrier was evaluated by measurement of the Evans blue (EB) content in the brain with spectrophotometer. The serum contents of interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) protein were determined by radioimmunoassay and ELISA assay. The expression of nuclear factor-κB (NF-κB) was evaluated with Western blot. **RESULTS** β-Aescin significantly reduced infarct volume ($P < 0.05$ or $P < 0.01$), ameliorated the neurological deficit and reduced the permeability of blood-brain barrier ($P < 0.05$). Pretreated with β-aescin 30 and 60 mg·kg^{-1}, the serum content of IL-8 and the expressions of TNF-α and NF-κB protein in brain tissue were significantly decreased ($P < 0.05$). **CONCLUSION** β-Aescin has protective effects on cerebral injury through inhibiting the expression and release of the inflammatory mediators after I/R injury.

Key words: β-aescin; interleukin-8; tumor necrosis factor; nuclear factor-κB; brain ischemia

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Brain injury can develop as a result of cerebral ischemia/reperfusion(I/R) due to stroke and other cardiovascular diseases. Stroke induces cerebral lesions through the combined action of multiple mechanisms, including excitotoxicity, free radical production, inflammation and apoptosis[1].

Brain inflammation has been implicated in the development of brain edema and secondary brain damage in I/R[2, 3]. In this process, adhesion molecules, cytokines and leukocyte chemo-attractants released/expressed at the site of blood-brain barrier play an important role in the process of inflammatory cells into the brain. Studies have shown that the inflammation in cerebral I/R is developed as an outcome of the two sequential, but closely linked processes: ① the activation of microglia and resident perivascular/parenchymal macrophages, and ② the mobilization and infiltration of peripheral inflammatory cells into the brain[4, 5]. Brain microvasular endothelium which performs the function of blood-brain barrier is an important responsive and regulatory component in cerebral inflammatory process. Over-expression and over-secretion of pro-inflammatory cytokines and chemotactic factors, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-8, etc., have led to microvascular vasoparalysis, abnormal blood rheology, and additional clotting of microcirculation, thus exacerbating initial ischemic damage. The expressions of these factors produced in brain microvascular endothelium are, at least in part, under the control of the ubiquitous nuclear factor-κB (NF-κB). For this reason, NF-κB has been considered as an important factor mediating inflammatory processes in brain injury following cerebral I/R[6-8]. NF-κB is a key transcription factor required in the expression of many pro-inflammatory genes[9]. Studies have shown that inhibition of an array of pro-inflammatory molecules can be achieved by suppressing NF-κB activation[10, 11].

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Aescin is the major active principle from *Aesculus hippocastanum* (Hippocastanaceae) the horse chestnut tree, which is a natural mixture of triterpene saponins exists in two forms: α- and β-aescin. β-Aescin appears to be the active component of the mixture and is the molecular form present in major available pharmaceutical products. A number of reports dating from the early 18th century have indicated therapeutic properties of β-aescin, such as anti-inflammatory, anti-edematous and veinotonic properties in some animal models[12,13]. It is also proved that β-aescin is capable of inhibiting peroxidation *in vitro* and suppressing production of free radicals in excess *in vivo*[14-16].

The aim of this study was to elucidate the beneficial effects of β-aescin on the cerebral injury in aspect of inhibiting the expressions of the pro-inflammatory cytokines. Based on the results of experiments[17,18], nimodipine has showed a beneficial effect on active treatment. So it was used as a control in this study.

1 MATERIALS AND METHODS

1.1 Materials

β-Aescin (sodium β-aescinate, purity: 98.5%, Wuhan Aimin Pharmaceutical Factory), it was dissolved in normal saline (NS); nimodipine injection (Bayer Company, Germany); 2,3,5-triphenyltetrazolium chloride (TTC, №20010201, Shanghai Chemical Agent Company); Evans blue (EB, Fluka); IL-8 assay kit (Beijing Beitudongya Bioengineering Institute, Beijing, China); TNF-α enzyme-linked immunosorbent assay (ELISA) kit, NF-κB primary antibody (rabbit polyclonal anti NF-κB) (Jinmei Bioengineering Institute, China).

1.2 Animal treatment and administration

Male Sprage-Dawley rats (Grade Ⅱ, Certificate № 19-050) weighing 230 – 280 g were obtained from the Experimental Animal Center of Tongji Medical College, which were kept at a constant room temperature of 22℃ under a 12 h light-dark cycle. Rats were divided into 6 groups, sham-operated and vehicle-treated I/R: NS 0.5 $mL \cdot kg^{-1}$, ig, for 7 d before ischemia; β-aescin-treated I/R: β-aescin 15, 30, 60 $mg \cdot kg^{-1}$, ig, for 7 d before ischemia; nimodipine-treated I/R: nimodipine 0.7 $mg \cdot kg^{-1}$, ip at 1 h preceding ischemia.

1.3 Cerebral ischemia/reperfusion procedure

At 1 h after administration, rats were anesthetized with 10% chloral hydrate (350 $mg \cdot kg^{-1}$) ip. Brain I/R injury was induced by a middle cerebral artery occlusion. Using introducing nylon suture method described previously[19], the right common carotid artery, external carotid artery (ECA) and internal carotid artery (ICA) was isolated *via* a ventral midline incision. A nylon monofilament, with its tip rounded by heating near a flame, was introduced into ECA lumen and advanced into the ICA in order to block the origin of the middle cerebral artery. The body temperature of the rats was maintained at 36.5 – 37.5℃ during the surgical procedure with an infra-red heat lamp. Sham-operated animals were not exposed to I/R. After 2 h of ischemia, the nylon suture was withdrawn to establish reperfusion. After arousal from anesthesia, the rats were returned to cages.

1.4 Measurement of infarct size, neurological outcome

After 22 h reperfusion, the neurological outcome was evaluated using 5 scores according to the Longa, *et al*[19] method: 0, no deficit; 1, failure to extend right paw; 2, circling to the right; 3, falling to the right; and 4, unable to walk spontaneously. Then the rats were anesthetized with 10% chloral hydrate (350 $mg \cdot kg^{-1}$) ip and then decapitated. The brains were removed for measurement of the infarct volume. The area of cerebral infarction was quantified with TTC staining. The brains were sectioned coronally with a brain slicer at 2 mm intervals from the frontal pole. All slices were incubated for 20 min in a 2% solution of TTC at 37℃ and fixed by immersion in 10% formaldehyde solution. With a computerized image analysis system (NIH Image, Version 1.61), the area of infarction of each section was determined. The total lesion volume was calculated by summing the

infarct area in each section and multiplying it by the distance between sections.

1.5 Analysis of the permeability of blood-brain barrier

The permeability of the blood-brain barrier was evaluated by the measurement of the EB content in the brain according to the method of Matsuo, *et al*[20]. Briefly, after 1.5 h reperfusion, the rats were treated with 2% EB (3 mg·kg^{-1}) iv. After 30 min, the rats were anesthetized with 10% chloral hydrate (350 mg·kg^{-1}) ip. 2% EB (50 mg·kg^{-1}) was administered to rats through a dissected femoral vein. After 30 min, thorax was opened and a needle was placed to the left heart ventricle. The needle was connected through a catheter with a system used for washing out the blood and EB contained in the vascular system and organs. This way was used for administering NS 300 mL·kg^{-1} to the vascular system. The excess of the NS together with EB washed out from the tissues, poured out through a prepared right heart atrium during 30 min. After opening the cranial vault, the brain was taken out, weighed (wet tissue), and then putted in a tube containing 1 mol·L^{-1} KOH 0.5 mL and stored at 37℃ over a night. Then 0.75 mL of 1 mol·L^{-1} H_3PO_4 and 2.25 mL acetone were added to the tube, and then centrifuged at 3500 × *g* for 15 min, 3 times. The supernatant was used to determine with spectrophotometer, λ = 620 nm. The amount of EB in 1 mg of the tissue was measured on the basis of the standard curve determined. The value of absorbance represented the content of EB in brain.

1.6 ELISA analysis of TNF-α

At the end of 3 h reperfusion following 2 h ischemia, the rats were decapitated and the brains of ischemic hemispheres were removed. The ischemic tissue was used for the assay of TNF-α. The procedure was according to a rat ELISA kit.

1.7 Radioimmunoassay (RIA) of IL-8

After 3 h reperfusion, 3 mL of blood was obtained from the common carotid artery. The samples were centrifuged at 3000 × *g* for 5 min and then the serums were isolated, stored at − 20℃ until required. The content of IL-8 in serum was measured according to the procedure described by the IL-8 assay kit.

1.8 Western blot analysis of NF-κB

After 22 h reperfusion, the rats were decapitated and the brains were removed. The ischemic hemispheres were used for the assay of the protein expression of NF-κB, and the sham-treated brains were used as control. Western blot analysis was performed as described previously[21]. The protein determination was performed according to Lowry, *et al* method. For the detection of the activated NF-κB in the brain tissue, 8 μg protein per lane were resolved on 12% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and placed in blocking buffer. The membranes were incubated with the primary anti-p65 antibody (rabbit polyclonal anti-NF-κB p65, 1∶200 dilution, Santa Cruz Biotechnology) in blocking buffer, and then the membranes were washed with phosphate buffered saline-Tween (PBS-T: 10 mmol·L^{-1} phosphate buffer pH 7.4, 150 mmol·L^{-1} NaCl, 0.05% Tween 20) for 30 min and incubated for 30 min with relevant horseradish peroxidase-conjugated secondary antibody (1∶6000 dilution). The membranes were washed again in PBS-T and immunoreactive protein bands were visualized using chemiluminescence detection system.

1.9 Statistics

Data were expressed as $\bar{x} \pm s$ and analyzed with Microsoft Excel 2002. Statistical analyses were performed by *t* test.

2 RESULTS

2.1 Effects of β-aescin on the area of cerebral damage, neurological score

Infarct tissue was visualized as an area of unstained part in vehicle group, in contrast to viable tissue, which stained red; while there was no infract part in sham-operated group. After treated with β-aescin the infarct volume was significantly reduced, and the neurological deficit was ameliorated (Tab 1).

Tab 1. Effects of β-aescin on the area of cerebral damage, neurological score after 2 h ischemia/22 h reperfusion (I/R) in rats

Group	Ratio of infarct area/%	Neurological score (in 5 score)
Sham	–	–
I/R + NS	28.3 ± 5.1	2.1 ± 1.4
I/R + β-aescin 15	22.7 ± 4.4	1.7 ± 0.6
I/R + β-aescin 30	17.0 ± 3.8*	0.8 ± 0.3**
I/R + β-aescin 60	5.6 ± 3.0**	0.8 ± 0.3**
I/R + Nim 0.7	19.2 ± 5.7*	1.9 ± 0.3

Brain I/R injury was induced by a middle cerebral artery occlusion. Sham and vehicle-operated rats: normal saline (NS) 0.5 mL·kg^{-1}, ig, for 7 d before ischemia; β-aescin-treated I/R: β-aescin 15, 30, 60 mg·kg^{-1}, ig, for 7 d before ischemia; Nim-treated I/R: nimodipine (Nim) 0.7 mg·kg^{-1}, ip, at 1 h preceding ischemia. $\bar{x} \pm s$, $n = 6$. * $P < 0.05$, ** $P < 0.01$, compared with I/R + NS.

2.2 Effects of β-aescin on blood-brain barrier

The data demonstrated that compared with sham-treated group, the content of EB in vehicle-treated group significantly increased after 2 h ischemia and 1.5 h reperfusion (Fig 1). β-Aescin 30 and 60 mg·kg^{-1} partially inhibited the increasing of the EB content induced by cerebral I/R, and there was no obvious difference between them.

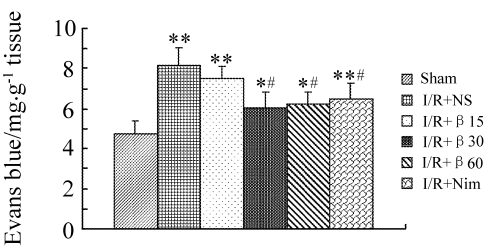

Fig 1. Effects of β-aescin on blood-brain barrier after 2 h ischemia and 1.5 h reperfusion. See legend of Tab 1 for rat treatments. β15, β30, β60: β-aescin 15, 30, 60 mg·kg^{-1}, respectively. $\bar{x} \pm s$, $n = 6$. * $P < 0.05$, ** $P < 0.01$, compared with sham group; # $P < 0.05$, ## $P < 0.01$, compared with I/R + NS group.

2.3 Effects of β-aescin on TNF-α protein in brain tissues and the content of IL-8 in serum

After 2 h ischemia and 3 h reperfusion, the contents of IL-8 in serum and TNF-α in brain were obviously increased in vehicle-treated group as compared with sham-treated group (Fig 2). β-Aescin at the dose of 30 mg·kg^{-1} partially decreased the content of IL-8, while completely at the dose of 60 mg·kg^{-1}. The content of TNF-α was obviously decreased after treated with β-aescin 30 and 60 mg·kg^{-1}.

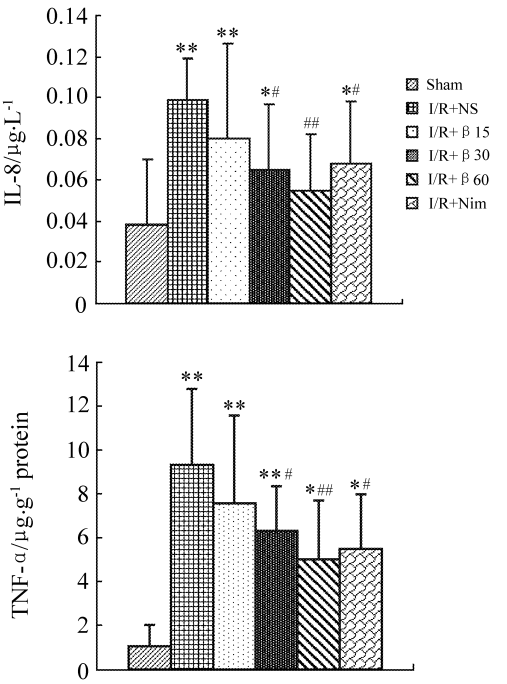

Fig 2. Effects of β-aescin on contents of interleukin-8 (IL-8) in serum and tumor necrosis factor-α (TNF-α) protein in brain tissue after 2 h ischemia/3 h reperfusion. See legend of Tab 1 for rat treatments. $\bar{x} \pm s$, $n = 6$. * $P < 0.05$, ** $P < 0.01$, compared with sham group; # $P < 0.05$, ## $P < 0.01$, compared with I/R + NS group.

2.4 Effects of β-aescin on the expression of NF-κB protein

NF-κB consists of a p50 and p65/RelA complex. In this study, Western blot assay was used to determine the NF-κB p65. The result showed that the expression of NF-κB protein was obviously increased in ischemic hemispheres in vehicle-treated rats; while was weak in sham-treated group. After treated with β-aescin 60 mg·kg^{-1}, NF-κB expression was significantly reduced compared with vehicle-treated group (Fig 3A, 3B).

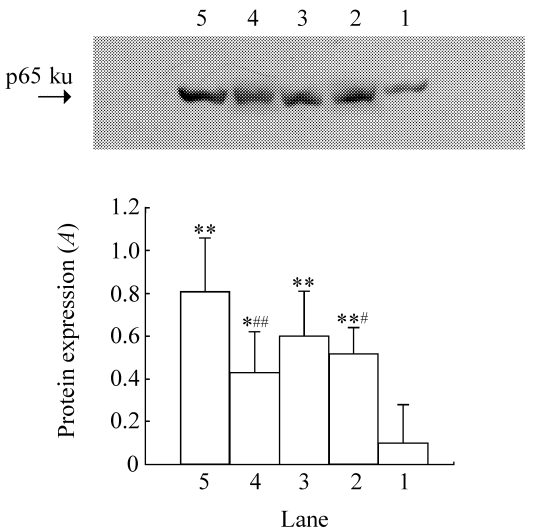

Fig 3. Effects of β-aescin on protein expression of nuclear factor-κB (NF-κB) in brain tissue after 2 h ischemia/3 h reperfusion. Lane 1: sham; lane 2: I/R + Nim; lanes 3, 4: I/R + β-aescin 30 and 60 mg·kg^{-1}, respectively; lane 5: I/R + NS. See legend of Tab 1 for rat treatments. $\bar{x} \pm s$, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with I/R + NS group.

3 DISCUSSION

As we have known, the development of ischemic brain inflammation is coordinated by the activation, expression, and secretion of numerous pro-inflammatory genes/mediators from both brain parenchymal and vascular cells. Chemokines (such as IL-8, TNF-α) are expressed in a variety of cells, including human peripheral and brain endothelium. After cerebral I/R, the contents of IL-8 and TNF-α obviously increase, causing the neutrophils and monocytes into the brain parenchyma and increasing the brain damage. Our study showed that β-aescin could significantly reduce cerebral infarct volume, edema and the damage on blood-brain barrier, decrease the release and expression of the inflammatory mediators (IL-8, TNF-α), and reduce the expression of NF-κB protein after cerebral I/R. These results were consistent with previous reports[22, 23]. In this study, nimodipine 0.7 mg·kg^{-1} (ip, one time) did not show more potent protective effects than β-aescin, especially in 30, 60 mg·kg^{-1} (ig, for 7 d).

In conclusion, sodium β-aescin possesses protective effect on cerebral injury induced by middle cerebral artery occlusion, which partially depends upon its inhibitory effects on inflammatory mediators and NF-κB activation. These experiments provide a further understanding of clinical therapeutic approach of β-aescin.

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β-七叶皂苷对大鼠脑缺血再灌注损伤时炎症反应的抑制作用

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摘要: 目的　研究 β-七叶皂苷对大鼠脑缺血再灌注损伤的保护作用是否与其抑制炎症反应有关。**方法**　大鼠缺血前分别给予 β-七叶皂苷 15, 30, 60 mg·kg^{-1}, ig, 7 d, 末次给药 1 h 后线栓法制备大脑中动脉阻断短暂局灶性脑缺血模型, 缺血 2 h, 再灌注 22 h, 评价神经功能状态和脑梗死范围; 用伊文思兰法测定脑缺血 2 h 再灌 3 h 后对血脑屏障的损伤程度。用放射免疫、酶联免疫分析及免疫印迹的方法测定大脑缺血区白介素-8(IL-8), 肿瘤坏死因子-α(TNF-α)及核因子-κB(NF-κB)的表达。**结果**　β-七叶皂苷能显著降低脑缺血再灌注后脑梗死体积, 改善神经功能症状, 其脑内 IL-8, TNF-α 的活性显著降低, NF-κB 的表达显著减少, 与模型组相比具有统计学显著意义。**结论**　β-七叶皂苷通过抑制炎性物质的表达和释放对大鼠脑缺血再灌注损伤具有明显保护作用。

关键词: β-七叶皂苷; 白介素-8; 肿瘤坏死因子; 核因子-κB; 脑缺血

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