Effects of β-aescin on apoptosis induced by transient focal cerebral ischemia in rats

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KEY WORDS apoptosis; beta-aescin; brain ischemia; cytochromes c; caspases; *bcl*-2 genes

ABSTRACT

AIM: To investigate the effects of β-aescin on apoptosis induced by transient focal brain ischemia in rats. **METHODS:** Rats were pretreated with β-aescin for 7 d and then subjected to brain ischemia/reperfusion (I/R) injury induced by a middle cerebral artery occlusion. After 2 h ischemia and 24 h reperfusion, Hematoxylin-Eosin (HE) staining, *in situ* end-labeling of nuclear DNA fragmentation (TUNEL) were employed to determine the level of apoptosis. The expressions of caspase-3 and Bcl-2 in the cortex were determined by immunohistochemistry and Western blot. The release of cytochrome c was analyzed by Western blot. **RESULTS:** The increased numbers of HE- and TUNEL-positive staining cells were significantly observed at 24 h after reperfusion. The immunoreactivity was inhibited by β-aescin (30, 60 mg/kg) ($P<0.01$ or $P<0.05$ *vs* vehicle-treated). After cerebral I/R, cytochrome c was released into the cytosol and caspase-3 was activated, whereas Bcl-2 expression was inhibited. β-Aescin (30, 60 mg/kg) markedly inhibited the expression of caspase-3 and the release of cytochrome c, and up-regulated the expression of Bcl-2 ($P<0.05$, $P<0.01$ *vs* vehicle-treated). **CONCLUSION:** β-Aescin could potently inhibit caspase-3 activation and the release of cytochrome c, increasing the expression of Bcl-2 after cerebral I/R in rats. These findings on the inhibitory effects of β-aescin on brain ischemic injury-induced apoptosis might have important theoretical basis for the treatment on ischemic cerebrovascular diseases.

INTRODUCTION

Aescin is the major active principle from *Aesculus hippocastanum (Hippocastanaceae),* the horse chestnut tree. The seeds of this tree contain a saponin mixture from which two crystalline products can be separated: aescin (haemolytic) and prosapogenin (non-haemolytic). Aescin is a natural mixture of triterpene saponins, which exists in two forms: α- and β-aescin. Studies found that β-aescin was the active component of the mixture which exists in molecular form in the major available pharmaceutical products[1] (Fig 1).

The β-aescin is widely used in the clinical therapy of vascular disorders and can attenuate neuronal damage because of its anti-inflammatory, anti-edematous, reducing the volume of cerebral infarct after brain damage, improving microvasculation in hemorrhage model, ameliorating mitochondria dysfunction, and microvascular protective activities[2,3]. The protective effects of β-aescin on cerebrovascular diseases have been previously reported and it was therefore of interest to study the mechanism of β-aescin on brain injury induced by cerebral ischemia/reperfusion (I/R).

Brain injury induces cerebral lesions through the

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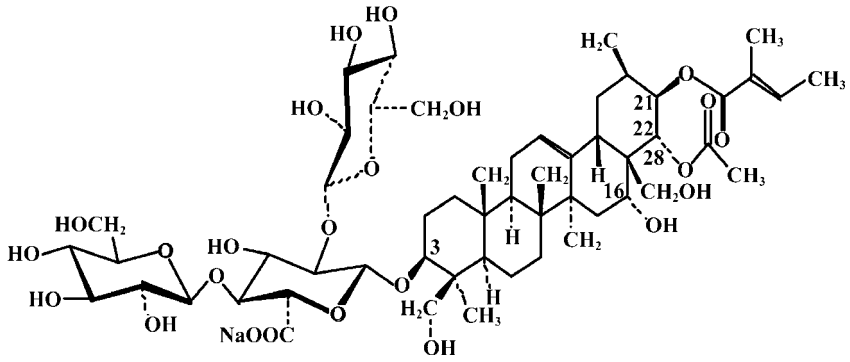

Fig 1. Chemical structure of β-aescin.

combined action of multiple mechanisms, including excitotoxicity, free radical production, inflammation, and apoptosis[4]. Caspases has been shown to be specifically involved in the initiation and execution phases of apoptosis[5,6]. Distinct pathways of initial caspase activation, death-receptor-dependent caspase-8 activation, and ROS-dependent caspase-9 activation via cytochrome c release from the mitochondria, all induce the proteolytic activation of caspase-3 and result in apoptotic death. Mitochondria are recognized as the main organelle in apoptosis by releasing apoptogenic proteins, such cytochrome c to the cytoplasm where it activates caspase-3[7,8]. The Bcl-2 family, consisting of antiapoptotic (eg, Bcl-2) and proapoptotic (eg, Bax and Bad) members, play important roles in the regulation of cell death[9-11]. The expression of Bcl-2 protein in the mitochondrial outer membrane inhibits cytochrome c translocation to cytosol and its overexpression prevents superoxide anion production[12-14], which is a critical step in the apoptotic process. Based on the evidence that β-aescin has the protective effect on mitochondrial damage, our current study is designed to investigate the effects of β-aescin on cerebral ischemia-induced apoptosis in rats.

MATERIALS AND METHODS

Drugs β-Aescin (purity: 98.5 %) was supplied by Wuhan Aiming Pharmaceutical Factory. It was dissolved in saline before use. All other chemicals and solvents were of analytical grade.

Transient focal cerebral ischemia Male Sprague-Dawley rats weighing 250-280 g (Experimental Animal Center of Tongji Medical College, Grate II, and Certificate No 19-050) were subjected to transient focal cerebral ischemia according to the method as described previously[15]. Briefly, rats were anesthetized with 10 % chloral hydrate (350 mg/kg) intraperitoneally (ip). 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 (Φ0.22-0.24 mm), with its tip rounded by heating near a flame, was introduced into ECA lumen and advanced into the ICA (about 18-22 mm deepness) in order to block the origin of the MCA. Sham-operated animals were not exposed to I/R. After 2 h ischemia, the nylon suture was withdrawn to establish reperfusion. Sham-operated rats were performed using the same surgical process but with no suture inserted. Rats were treated with saline and β-aescin 15, 30, and 60 mg/kg (po) once daily for 7 d before ischemia.

Neurological deficits After 2 h ischemia and 24 h reperfusion, the neurological deficits were evaluated by a four-tiered grading system, which was developed by Bederson *et al*[16].

Hematoxylin-Eosin (HE) staining, immunohistochemistry After 2 h ischemia and 24 h reperfusion 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 cry-protected in 25 % sucrose in PBS. Then the brains were coronally sectioned on a cryostat at 20 μm thickness or paraffin embedded and sliced on a microtome at 6 μm thickness. The sections between 3 and 4 mm posterior of the bregma were used for this study. For histological assessment of damage to the cortex and striatum, the paraffin-embedded brain sections were stained with HE. For assay of the expressions of caspase-3 and Bcl-2, the free-floating vibratome sections were processed immunohistochemically. Briefly,

an ABC kit (Zhongshan Biotechnology Company, Beijing) was used to localize the primary antibody. The diaminobenzidine (DAB) kit (Zhongshan Biotechnology Company, Beijing) was used to visualize the catalyzed peroxidase-reaction product. The primary antibodies were the rabbit polyclonal antibody against caspase-3 (Santa Cruz Biotechnology, at 1:50 dilution) or the mouse monoclonal antibody against Bcl-2 (Santa Cruz Biotechnology, at 1:100 dilution). At the same time, the negative controls were performed without the primary antibodies.

Terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated UTP nick end labeling (TUNEL) assay The sections were placed in TdT buffer for 15 min, followed reaction with TdT enzyme and biotinylated 11- dUTP at 37 ºC for 60 min. An ABC solution was applied to the sections for 30 min. Then the sections were stained with DAB. The negative controls were also performed.

Western blot At 24 h after reperfusion or sham-operation, the brains were removed and divided into ipsilateral (infarct) and contralateral (noninfarct) hemispheres. The ipsilateral tissue was used for the assay of the protein expression of Bcl-2, caspase-3, and the release of cytochrome c.

The tissues were homogenized in ice-cold buffer (Tris 50 mmol/L, pH 7.4, NaCl 150 mmol/L, 0.5 % Triton X-100, edetic acid 1 mmol/L, phenylmethylsulfony fluoride 1 mol/L, and aprotinin 5 mg/L), and centrifuged at 14 000×*g* at 4 ºC for 30 min. The supernatant were then collected as total proteins. The preparation of the protein extraction of the mitochondrial and the cytosolic fraction was performed as the method described previously[17].

The obtained protein samples were denatured in 15 % SDS polyacrylamide gel. After electrophoresis, the proteins were electrically transferred to a nitrocellulose membrane. The membranes were incubated in 5 % milk TBST for at 4 ºC overnight, and detected with the primary antibodies: the mouse monoclonal antibody against cytochrome c (PharMingen, 1:500 dilution), the rabbit polyclonal antibody against caspase-3 (Santa Cruz Biotechnology, 1:200), and the mouse monoclonal antibody against Bcl-2 (Santa Cruz Biotechnology, 1:200 dilution). After washing with TBST, the membranes were incubated with the secondary antibodies (horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG) at a room temperature for 1 h. The gels were scanned and quantified by Image Quant V1.19 (Molecular Dynamics, Sunnyvale, CA). The levels of cytochrome c, caspase-3, Bcl-2, and β-actin were quantified by Image Quant programmer, and the relative amount was obtained after normalization with β-actin values in the same lane.

Statistics analysis Data were expressed as mean±SD and analyzed with Microsoft Excel 2002. Statistical analysis were performed by *t*-test. $P<0.05$ was considered to be significant. For quantitative analysis of caspase-3 and Bcl-2 and the TUNEL-positive cell, the stained-sections were observed under light microscope at magnification of 10±20 (OLYMPUS BX51) and analyzed by computerized imagine analysis system (HPIAS-1000, Champion Imagine).

RESULTS

Effects of β-aescin on neurological deficits Compared with the vehicle-treated group, the neurological deficit in rats pretreated with β-aescin (30, 60 mg/kg) decreased significantly ($P<0.05$). But there was no obvious deference between the β-aescin 15 mg/kg group and the vehicle-treated group ($P<0.05$, Fig 2).

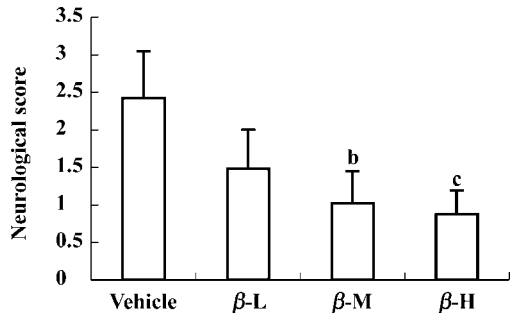

Fig 2. Effects of β-aescin on neurological deficits after 2 h of ischemia and 24 h of reperfusion in rats. Vehicle: Vehicle-operated group; β-L: β-aescin-operated group (15 mg/kg); β-M: β-aescin-operated group (30 mg/kg); β-H: β-aescin-operated group (60 mg/kg). *n*=6. Mean± SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* vehicle.

Effects on HE and TUNEL staining DNA fragments reached the peak between 24-48 h after cerebral I/R[18]. So after ischemia/reperfusion, HE- and TUNEL-staining were employed to study the effect of β-aescin on the level of apoptosis. In vehicle-treated group, the features of the neurons in the cortex and striatum appeared obviously as following: the cell size decreasing, the cytoplasm condensing, aggregation of chromatin into dense staining. The cells were scored as apoptosis

when they were TUNEL-positive staining (brown staining) and the nuclear chromatin condensing. The above features were obviously observed in the vehicle-treated group (Fig 3B, 3F). However, the cells without nuclei TUNEL stained were localized in the sham-operated group (Fig 3E) and the contralateral hemisphere of ischemic rats. β-Aescin (30, 60 mg/kg) obviously reduced the damage of the neurons (Fig 3C, 3D, 3G, 3H). In the vehicle-treated group, the number of the TUNEL-positive cells significantly increased in the cortex[(2.51±0.52)×10/mm^2] and striatum [(2.68±0.56) ×10/ mm^2] in the ischemic hemisphere as compared with the sham-operated group. Pretreated with β-aescin (30, 60 mg/kg), the number of TUNEL-positive cells

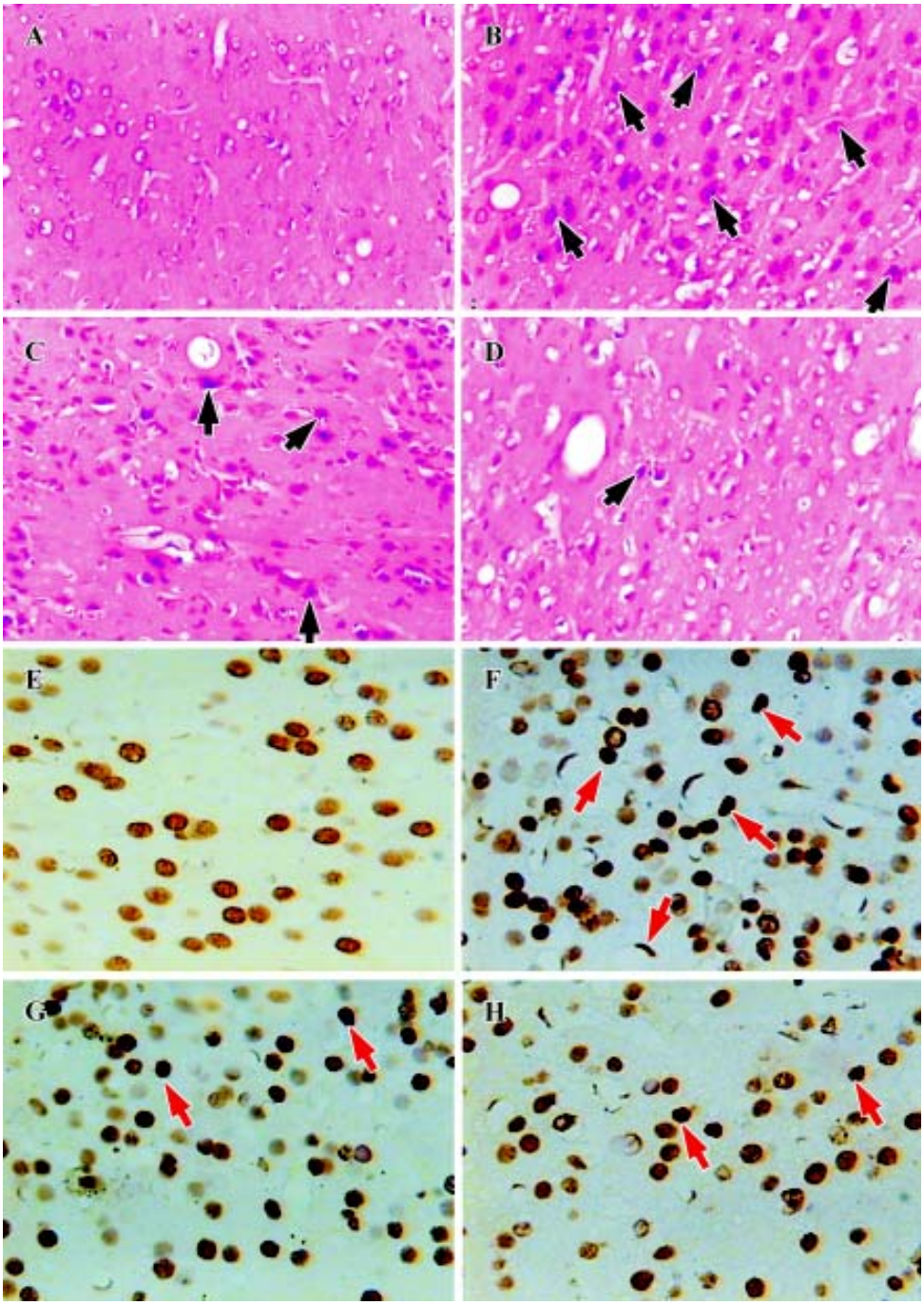

Fig 3. Representative photomicrographs of HE staining (A-D) and TUNEL analysis (F-H) in cortex in coronal cerebral sections between 3 and 4 mm posterior of the bregma after 2 h ischemia/24 h reperfusion in rats treated with or without β-aescin. (A, E): Sham; (B, F): vehicle; (C, G): β-aescin (30 mg/kg); (D, H): β-aescin (60 mg/kg). Arrows in B, F: HE and TUNEL positive-staining cells. *n*=6.

remarkably decreased (Fig 4).

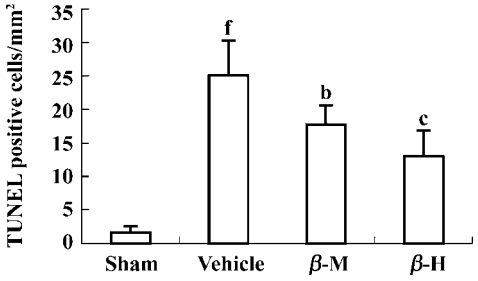

Fig 4. Quantitative analysis of the effects of β-aescin on the TUNEL-positive cells in the cortex in coronal cerebral sections between 3 and 4 mm posterior of the bregma after 2 h ischemia/24 h reperfusion in rats. Four to five microscopic fields per region on each section were analyzed. β-M and -H: β-aescin 30, 60 mg/kg. *n*=6. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* vehicle. $^{f}P<0.01$ *vs* Sham.

Effects of β-aescin on the release of cytochrome c The effects of β-aescin on cytochrome c were observed after 24 h reperfusion by Western blot[18,19] (Fig 5A, 5B). The expression of cytochrome c in mitochondria were obviously decreased in the vehicle treated rat brain tissue, with corresponding increased in cytosolic fraction after cerebral I/R (Fig 5A, Lane 2); whilst strong expressions were obviously observed in mitochondria in the sham-operated group, but a weak protein expression in cytosolic fraction (Lane 2). After treated with β-aescin (30, 60 mg/ kg), the immunoreactivity of cytochrome c was markedly decreased in cytosolic fraction as compared with the vehicle-treated group (Lane 3 to Lane 4, $P<0.05$ or $P<0.01$). At the meantime, a consistent amount of β-actin immunostaining was detected at the bottom panel, which suggested that the amount of the loaded protein at each lane was consistent.

Effects of β-aescin on the expression of caspase-3 and Bcl-2 The caspase-3 protease in cell exists as the precursor (M_r=32 000), which is cleaved into two subunits when activated: p17 (M_r=17 000) and p12 (M_r=12 000). The antibody against caspase-3 we used in this study recognized both the inactive precursor and p17 in the rat brain protein extracts at 24 h after reperfusion. The sample obtained from the sham-operated rat brain tissue showed strong protein expression of the precursor but very low level expression of the p17 subunit (Fig 6A, Lane 1); whereas the level of the p17 subunit significantly increased in the ischemic hemisphere ($P<0.01$, *vs* sham-operated) (Fig 6A, Lane 2). On the other hand, the protein expression of Bcl-2 in ischemic hemisphere was observed weakly or diffusely in the vehicle-treated rat brain after cerebral I/R (Fig 6B, Lane 2). These results were consistent with previous reports[20, 21]. After treated with β-aescin (30, 60 mg/kg), the immunoreactivity of the p17 subunit was markedly decreased (Fig 6A, Lane 3, 4), whiles the protein expression of Bcl-2 was increased in the ischemic hemisphere (Fig 6B, Lane 3, 4) (*vs* vehicle-treated, $P<0.05$ or $P<0.01$). The similar findings were observed by method of immunohistochemistry: remarkable immunoreactivity of caspase-3 was observed (Fig 7B) and with corresponding low expression of Bcl-2 (Fig 7F) in the ischemic hemisphere of the vehicle-treated rat brain sections. After treated with β-aescin (30, 60 mg/kg), the decreased immunoreactivity of caspase-3 and the up-regulated expression of Bcl-2 were obviously showed (*vs* vehicle, $P<0.05$ or $P<0.01$, Fig 7C, 7D, 7G, 7H). In the above analysis, a consistent

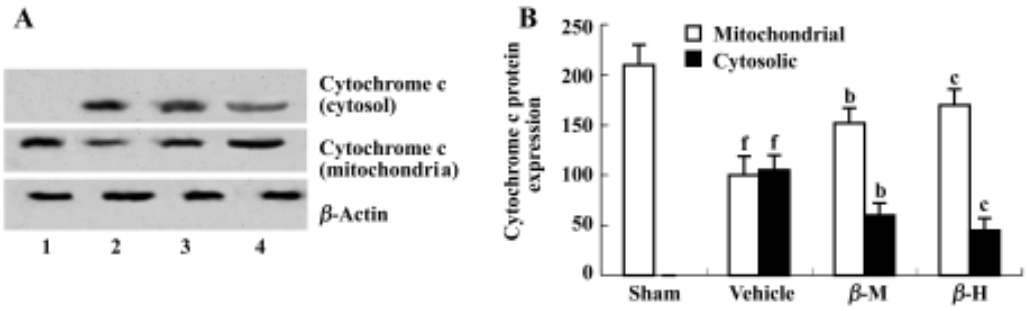

Fig 5. (A) Western blot analysis of β-aescin on the release of cytochrome c in cytosolic and mitochondrial fractions after 2 h ischemia and 24 h reperfusion in rats. Lane 1: Sham; Lane 2: vehicle; Lane 3 and Lane 4: β-aescin (30, 60 mg/kg); β-actin as an internal control. (B) Quantitative analysis of β-aescin on the release of cytochrome c (the relative abundance of the immunostaining). Cytochrpme c levels are expressed as % of the absorbance of the band. β-M and -H: β-aescin (30, 60 mg/kg). *n*=6. Mean±SD. $^{b}P < 0.05$, $^{c}P<0.01$ *vs* vehicle. $^{f}P<0.01$ *vs* Sham.

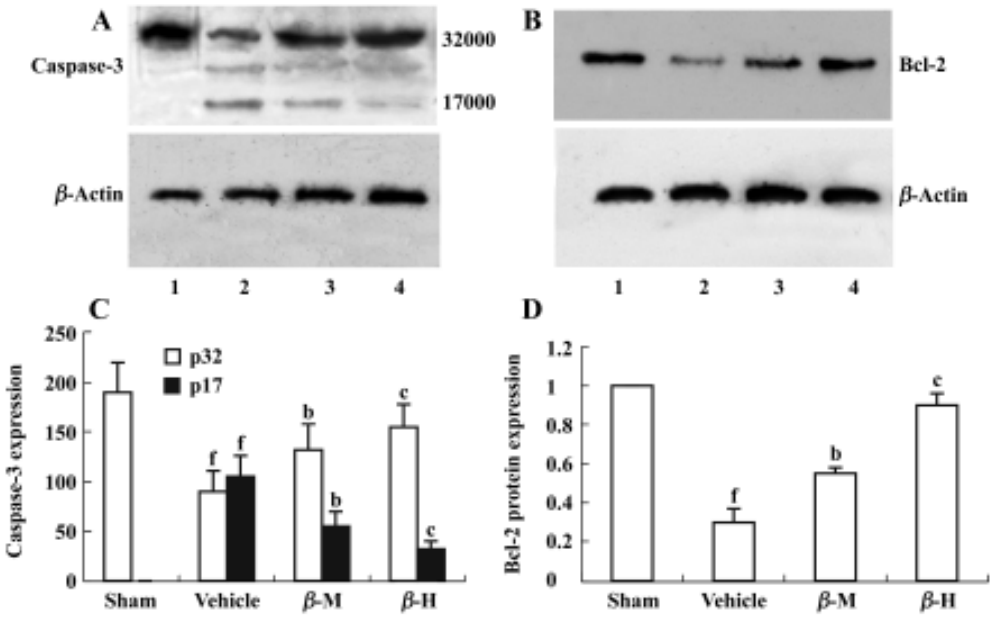

Fig 6. Western blot analysis of β-aescin on the expression of caspase-3 (A) and Bcl-2 (B) after 2 h ischemia and 24 h reperfusion in rats. Lane 1: sham; Lane 2: vehicle; Lane 3 and Lane 4: β-aescin (30, 60 mg/ kg); β-actin as an internal control. (C-D) Quantitative analysis of β-aescin on the expression of caspase-3 (C) and Bcl-2 (D) (the relative abundance of the immunostaining) determined by Image Quant programmer. The levels of caspase-3 and Bcl-2 are expressed as % of *A*. β-M and -H: β-aescin-operated (30, 60 mg/ kg). *n*=6. Mean±SD. ^{b}P <0.05, ^{c}P<0.01 *vs* vehicle. ^{f}P<0.01 *vs* Sham.

amount of β-actin immunostaining was detected at the bottom panel, which suggested that the amount of the loaded protein at each lane was consistent.

DISCUSSION

In the present study, we observed the effects of β-aescin on the cell apoptosis through delaying caspase-3 activation by upregulating Bcl-2 expression after 2 h of ischemia following 24 h of reperfusion in rat brain. The results showed that β-aescin at doses of 30, 60 mg/kg exerted potent protection on brain injury induced by MCAO following reperfusion.

A number of studies have documented that apoptosis contributes to the development of ischemic infarction with DNA fragmentation, which prominently occurs in the penumbral zone with moderately reduced cerebral blood flow[22,23]. In the penumbral region, blood flow is reduced to a critical level during occlusion of MCA, but reperfusion after ischemia provides an excess of oxygen with restored blood flow, leading to not only sustaining neuronal viability but also catalyzing enzymatic oxidative reaction to produce ROS, triggering apoptosis or laddered DNA fragmentation[24]. The improvement of apoptosis in tissue injury induced by cerebral ischemia is currently discussed[25,26], which widely occurs in human ischemic stroke. The previous studies[20, 21] have found that DNA fragmentation peaked between 24-48 h after cerebral I/R, and most cells exhibiting DNA fragmentation were neurons. In our current study, the effects of β-aescin on cerebral ischemia-induced apoptosis were investigated with HE and TUNEL staining assay at 24 h reperfusion. A remarkable amount of HE and TUNEL-positive staining cells were detected after MCAO. After treated with β-aescin, especially at doses of 30, 60 mg/ kg, the level of DNA fragmentation in the cortex and the striatum in the ischemic hemisphere was obviously decreased. The above results suggested that transient global ischemia resulted in cell apoptosis in selectively vulnerable brain regions and β-aescin could markedly decrease the number of TUNEL-positive cells and inhibit DNA fragmentation after transient focal cerebral ischemia. Therefore, the present study implicated that the protective effect of β-aescin on brain ischemia-induced injury partially due to the inhibition of apoptotic death.

The complex processes of apoptosis implicated the activation of cysteine protease of the caspases family, alterations in plasma membranes phospholipids, and nuclear DNA condensation and fragmentation[27]. In the process of apoptosis, cytochrome c is released from mitochondria to cytosol and caspase-3 is activated. On

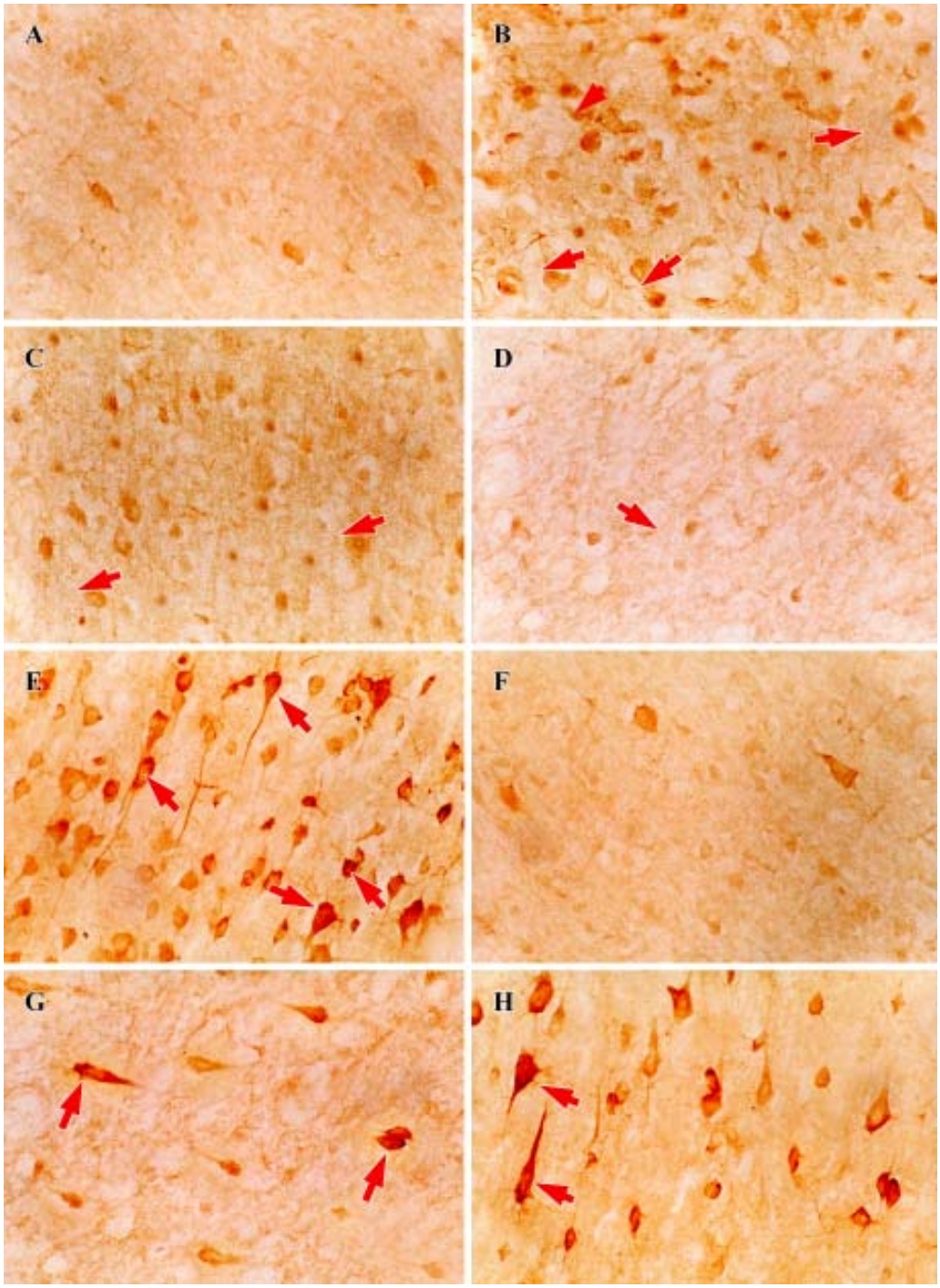

Fig 7. Representative photomicrographs of caspase-3 (A-D) and Bcl-2 (F-H) immunohistochemistry in cortex in coronal cerebral sections between 3 and 4 mm posterior of the bregma after 2 h-ischemia/24 h-reperfusion in rats treated with or without β-aescin. (A, E): sham; (B, F): vehicle; (C, G): β-aescin (30 mg/kg); (D, H): β-aescin (60 mg/kg). Arrows: caspase-3 and Bcl-2 immunoreactivity cells. *n*=6. ×200.

the other hand, over-expression of Bcl-2 protects neurons from ischemia-induced cell death[28]. In this study, the expressions of caspase-3 and Bcl-2 in the process of cerebral ischemia-induced apoptosis were determined before and after treatment with β-aescin after 2 h ischemia and 24 h reperfusion. The results showed that the remarkable release of cytochrome c was found at 24 h reperfusion. At the mean time, the activation of caspase-3 and the reduction of Bcl-2 expression were also detected in ischemic hemisphere after reperfusion. After β-aescin treatment, the release of cytochrome c and the activation of caspase-3 were markedly reduced. We also detected that this inhibitory effect of the release of cytochrome c and the activation of caspase-3

was paralleled by an increase in Bcl-2 levels in our work. Although it did not prove the hypothesis that the inhibition of β-aescin on cerebral ischemia-induced apoptosis directly contributed to decrease in the release of cytochrome c and the expression of caspase-3, and its upregulation on Bcl-2 protein expression, the present results at least implicated the possibility that the β-aescin might suppress apoptosis partially due to the decrease in the release of cytochrome c, the inhibition of caspase-3 and the increase of Bcl-2 expression. These experiments would provide important information for the further mechanism study of β-aescin on cerebral ischemia-induced apoptosis.

Taken together, pretreatment with β-aescin at the doses of 30, 60 mg/kg before cerebral ischemia, markedly inhibited the release of cytochrome c, decreased the activation of caspase-3 protein, upregulated Bcl-2 expression and reduced DNA fragmentation after MCAO following reperfusion. Our findings would provide a therapeutic basis for its clinical treatment on ischemic cerebrovascular diseases.

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