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Original Article

The protective effect of cardamonin on the factors involved in delayed cerebral vasospasm in a rat model of subarachnoid hemorrhage

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Abstract: Delayed cerebral vasospasms (DCVS) may affect the prognosis of patients after subarachnoid hemorrhage (SAH), but available preventive approaches are inefficient. The objective of this study was to explore the effects of cardamonin treatment on factors associated with the occurrence of DCVS after SAH. Rat models of SAH were created using the internal carotid artery puncture method. Rats were randomized into four groups: SAH (n = 10), SAH + vehicle (saline solution) group (n = 10), SAH + cardamonin group (n = 10), and a control (sham operation) group (n = 6). H&E staining was used to determine the wall thickness of the basilar artery. Immunohistochemistry was used to detect p-AKT and alpha smooth muscle actin (α-SMA). Immunofluorescence was used to detect the changes in C-myc expression. The TUNEL assay was used to detect apoptosis. Basilar artery wall thickness in the SAH + cardamonin and control groups were significantly lower than in the SAH group and SAH + vehicle groups (all $P < 0.01$). Apoptosis and the expression of p-AKT and C-myc in the SAH + cardamonin group were significantly lower than in the SAH and SAH + vehicle groups ($P < 0.05$), while α-SMA expression was higher than in the SAH and SAH + vehicle groups ($P < 0.01$). Cardamonin seems to alleviate cerebral vasospasms after SAH. These effects may involve the inhibition of p-AKT, C-myc expression and apoptosis, and the increase of α-SMA expression.

Keywords: Cardamonin, delayed cerebral vascular vasospasm, Akt, c-myc, α-smooth muscle actin, apoptosis

Introduction

Subarachnoid hemorrhage (SAH) is caused by the presence of blood in the cavum subarachnoidale, usually due to the rupture of an intracranial aneurysm [1, 2]. It is associated with high morbidity and mortality [1, 2]. About 16.7% of patients die during the acute period of SAH, which may be related to the amount of bleeding. After SAH, re-bleeding because of intracranial aneurysm re-rupture, hydrocephalus, and delayed cerebral ischemia (DCI) are factors that affect the clinical prognosis of SAH. DCI is also strongly associated with the prognosis of SAH [3, 4]. The incidence of DCI in all patients with SAH is 30% and 50% of them have a poor prognosis [5]. Delayed cerebral vascular vasospasms (DCVS) are considered to be the cause of high mortality and ischemic complications in patients with SAH [3, 4]. DCVS occur within 4-10 days after SAH, consistent with the occurrence of delayed cerebral ischemia [3, 4]. The specific mechanisms of DCVS are still not clear, but the proliferation, transformation, and apoptosis of vascular smooth muscle cells (VSMCs) might play an important role in DCVS [6].

Cardamonin (2,4-dihydroxy-6-methoxyphenylcone) is mainly found in cardamom spice, but it's also found in many other species of edible plants [7]. Cardamonin has anti-inflammatory activities exerted via the NF-κB pathway [8, 9]. A previous study revealed that cardamonin decreased the inflammatory response during sepsis by downregulating TNF-α, interleukin (IL)-1β, and IL-6 [10]. Cardamonin also inhibits platelet aggregation and has potent antioxidant activity [7]. Cardamonin has obvious vasodilatory effects by increasing NO levels and decreasing protein kinase C, which can induce

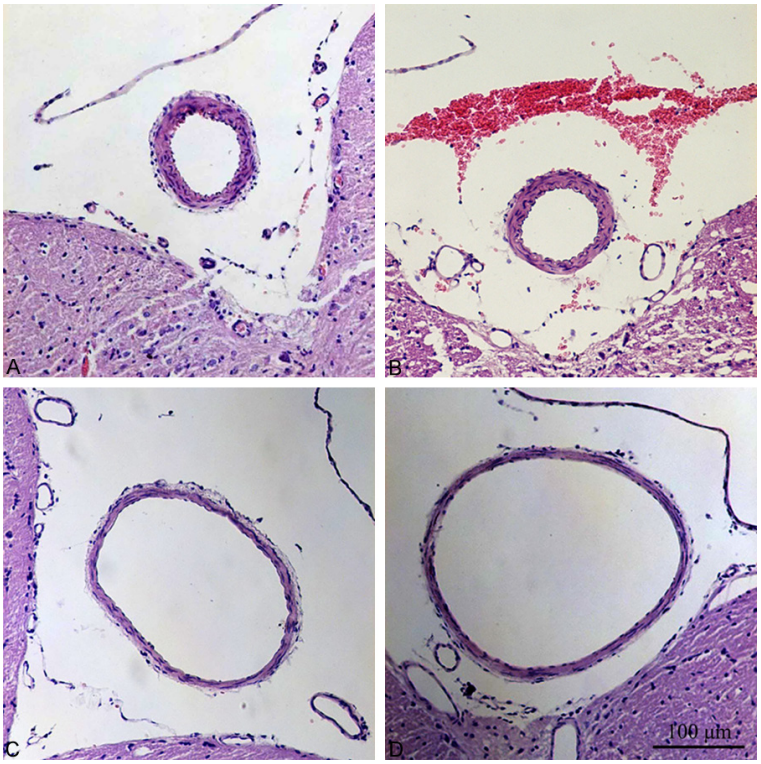

Figure 1. HE staining of the basilar artery to determine the mean wall thicknesses resulting from different treatments. A. SAH group. B. SAH + vehicle group. C. SAH + cardamonin group. D. Control group. Scale bar = 100 μm.

blood vessel contraction [9]. Animal experiments showed that cardamonin can inhibit the proliferation of VSMCs, which is induced by insulin via the mammalian target of rapamycin (mTOR)/translation control proteins p70 ribosomal S6 kinase (P70S6K1)/eukaryotic initiation factor 4E binding protein 1 (4E-BP1) signal transduction pathway [11]. Of significance for the potential prevention of DCVS, cardamonin may act as a vasodilator by inhibiting the voltage-dependent Cav1.2 channels, as well as through endothelium-independent and -dependent relaxation effects [12].

Therefore, the aim of the present study was to assess the effects of cardamonin on the proliferation, transformation, Akt signal transduction pathway, and apoptosis of VSMCs in a rat model of SAH. The results could provide new insights for the prevention of DCVS after SAH.

Materials and methods

Animals

Thirty-six healthy adult male SD rats (250-300 g) were obtained from the Experimental Animal Center of Chinese PLA Military Academy of Medical Sciences. The rats were divided into the SAH (n = 10), SAH + vehicle (n = 10), SAH + cardamonin (n = 10), and control (sham operation) (n = 6) groups.

The internal carotid SAH rat model was generated. In brief, the rats were anesthetized with 5% chloral hydrate (5.4 ml/kg, i.p.). Their legs were fixed and the neck hairs were shaved. A partial incision was made on the left side of the median line of the neck, and the left common carotid artery, jugular artery, and external carotid artery were exposed and isolated. Then, the common carotid artery was clamped and the left external carotid artery was cut distally and reflected caudally. A 3-0 wire was inserted into the stump to enter the internal carotid artery until resistance was felt, and then a further 2 mm to perforate the artery. The wire was removed to induce hemorrhage. The stump was closed and the clamp was removed. The time interval between occlusion and reperfusion was 2 min. The sham operation was done following the same procedure but without the hemorrhage.

Post-surgery, the rats in the SAH + cardamonin group received intraperitoneal daily injections of cardamonin (7.5 mg/kg; Sima Technology Co. Ltd., Tianjin, China) dissolved in vehicle solution (deionized water: Tween 80: ethanol = 7.2:1:1.8). The rats in the SAH + vehicle group received the vehicle solution only.

The rats were euthanized 7 days after surgery. In each, the chest was opened to expose the heart. A needle was placed in the left ventricle and the right atrium was perforated. A hemostatic pump was used to infuse about 150 ml of PBS and 60 ml of 4% paraformaldehyde. The brains were removed, placed in 4% paraformaldehyde for 24 h, and embedded in paraffin. Sections from the middle of the basilar artery were taken for analysis. Image Pro-Plus 6.0 software was used to determine the wall thick-

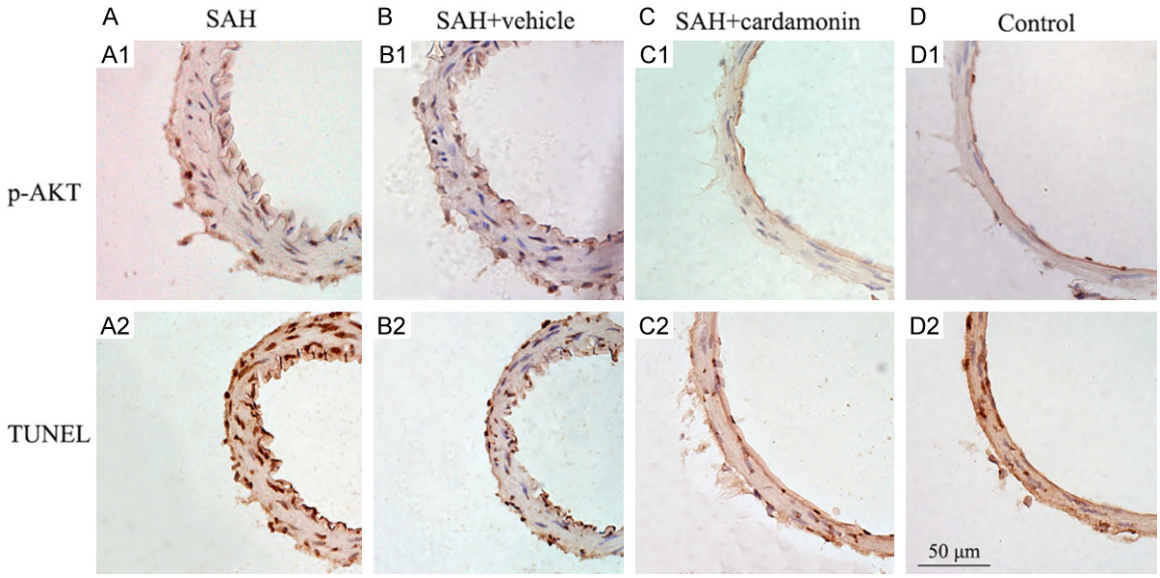

Figure 2. Immunohistochemistry for p-AKT (top panel) and TUNEL staining (bottom panel) of basilar artery sections following different treatments. A1 and A2. SAH group. B1 and B2. SAH + vehicle group. C1 and C2. SAH + cardamonin group. D1 and D2. Control group. Scale bar = 50 μm.

ness of the basilar artery. This study was approved by the Ethics Committee of the Affiliated Hospital of Logistics, University of Chinese People's Armed Police Forces, Tianjin 300162, China.

Immunohistochemistry

Tissue sections were dewaxed in xylene and hydrated in an ethanol gradient. Endogenous peroxidase activity was quenched by 3% H_2O_2. After high-pressure hot fixation for 5 min, the tissue sections were cooled at room temperature for 1 h and washed with PBS three times, 3 min/time. Then, 2% goat serum was used to block unspecific signals for 15 min at room temperature. The sections were incubated with a primary antibody against p-AKT (ser473) (1:100; Abcam, Cambridge, United Kingdom, monoclonal antibody) at 4°C overnight. Afterwards, the secondary antibody was incubated for 15 min. Next, diaminobenzidine was used as the chromogen, and the tissue sections were counterstained with hematoxylin, dehydrated, xylene, and mounted with neutral resin. The slides were observed under a microscope (Nikon, Tokyo, Japan).

Immunofluorescence

The tissue sections were conventionally dewaxed in deionized water for 5 min, and incubated with 0.1% Triton-100 for 10 min. After fixation under high pressure at 95°C for 5 min, the tissue sections were treated with 2% goat serum for 20 min at room temperature. Then, primary antibodies against C-myc rabbit (1:200; Abcam, Cambridge, United Kingdom, monoclonal antibody) and α-SMA (1:200, Abcam, Cambridge, United Kingdom, monoclonal antibody) were incubated at 4°C for more than 16 h. Then, FITC-labeled goat anti-rabbit and goat anti-mouse TRITC labeled secondary antibodies (1:100) were added and incubated for 1 h at room temperature, and the sections were counterstained with DAPI. After PBS washing (5 min/times, four times), a 50% glycerol carbonate buffer was added to the tissue sections, which were observed under an ECLIPSE 80i fluorescent microscope (Nikon, Tokyo, Japan).

TUNEL assay

Tissue sections were dewaxed and endogenous peroxidase activity was quenched by 3% H_2O_2 in deionized water for 10 min. The sections were rapidly heated in a 0.1 M sodium citrate buffer in a microwave oven for 5 min followed by PBS washes (5 min/times). Then, the TUNEL reagent was added. A paraffin film was used to prevent evaporation and the samples were incubated at 37°C for 1 h. Afterwards, POD was added to the slides, which were sealed with a paraffin film to prevent evaporation, and incu-

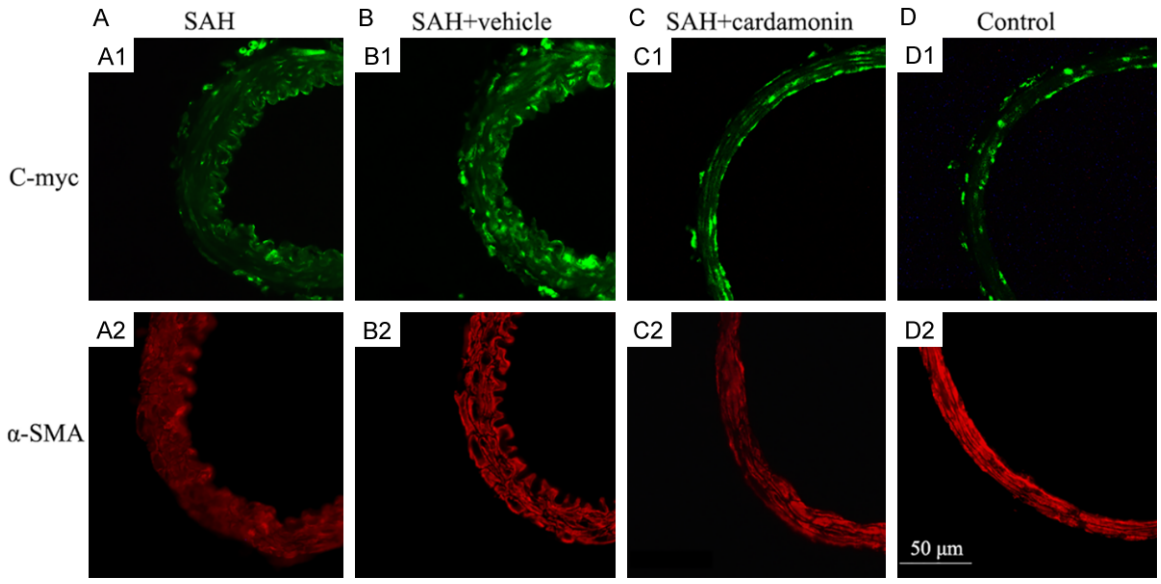

Figure 3. Immunofluorescence of C-myc (upper panel, green) and α-SMA (bottom panel, red) expression of basilar artery sections following different treatments. A1 and A2. SAH group. B1 and B2. SAH + vehicle group. C1 and C2. SAH + cardamonin group. D1 and D2. Control group. Scale bar = 50 μm.

bated at 37°C for 30 min. Next, a DAB chromogenic agent was added to the slides for 10 min before termination with running water. The slides were counterstained with hematoxylin, dehydrated, treated with xylene, and mounted with neutral resin. The tissue sections were observed under an ECLIPSE 80i fluorescent microscope (Nikon, Tokyo, Japan).

Statistical analysis

Data were presented as the means ± standard errors. Continuous data were compared using ANOVA followed by the LSD post-hoc test. All analyses were performed using SPSS 19.0 (IBM, Armonk, NY, USA). *P*-values < 0.05 were considered statistically significant.

Results

Cardamonin reduced the basilar artery wall thickness after SAH

The histology was evaluated post SAH model establishment. HE staining showed that basilar artery wall thickness in the SAH group was 20.53 ± 2.97 μm, which was comparable to the wall thickness in the SAH + vehicle group (19.56 ± 0.64 μm) (P = 0.41). Meanwhile, the basilar artery wall thickness in the SAH + cardamonin and the control groups were significantly lower than in the SAH group and the SAH + vehicle groups (all P < 0.01, **Figure 1**).

Cardamonin decreased p-Akt expression and apoptosis after SAH

Next, the expression of p-AKT was evaluated. Immunohistochemistry showed that, compared with the rats in the control group, the expression of p-AKT (ser473) was significantly higher in the SAH rats (P < 0.05), while p-AKT (ser473) in the SAH + cardamonin group was significantly lower compared with the SAH and SAH + vehicle groups (all P < 0.05). As expected, there was no significant difference between the SAH and SAH + vehicle groups (**Figure 2**, top panel).

Then, the apoptosis ratio was determined using the TUNEL assay, which revealed that compared with the control group, the apoptosis rate was significantly higher in the SAH rats (P < 0.05), while the cardamonin significantly decreased the apoptosis ratio compared with the rats treated with the vehicle (P < 0.05). There was no significant difference between the SAH and SAH + vehicle groups (**Figure 2**, bottom panel).

Cardamonin decreased C-myc expression and increased α-SMA expression after SAH

Finally, the expressions of C-myc and α-SMA were evaluated by immunofluorescence. Compared with the control group, the C-myc expression in the SAH group was significantly higher and the α-SMA expression was lower (P < 0.05).

Compared with the SAH group, the C-myc expression in the SAH + cardamonin group was lower while the α-SMA expression was higher ($P < 0.05$) (**Figure 3**).

Discussion

The aim of the present study was to assess the effects of cardamonin on the proliferation, transformation, and apoptosis of VSMCs in a rat model of SAH. Our results showed that the injection of cardamonin after SAH reduced the basilar artery wall thickness, decreased p-Akt and C-myc expression, decreased VSMC apoptosis, and increased α-SMA expression after SAH. These results might provide new insights for the prevention of DCVS after SAH.

Currently, the main approaches for the prevention of DCVS after SAH include treatments aiming to control hypertension, high blood volume, and blood dilution using calcium channel antagonists, Mg ions, ET-1 antagonists, vasodilators, nitric oxide, antioxidants, anti-inflammatory treatment, anti-platelet aggregation, micro-emboli prevention, and statins [13]. Nevertheless, the clinical efficacy of these approaches is not satisfactory [13].

The pathogenesis of DCVS still remains unclear. Multiple mechanisms might be involved such as inflammation pathway activation, oxidative stress, vascular proliferation, and apoptosis [14]. Normal contractility of VSMCs plays an important role in the regulation of vascular contraction and relaxation [15]. Transformation of VSMCs from the contractile phenotype to the endocrine phenotype and VSMC apoptosis are currently the two most studied pathways for the occurrence of DCVS [16, 17]. Under many pathological conditions, VSMCs proliferate, resulting in blood vessel wall thickening and stenosis [18]. In the present study, α-SMA was used as a marker of VSMC transformation. The results showed that SAH decreased the expression of α-SMA, while cardamonin partly restored this expression. This suggests that cardamonin could be effective to prevent the transformation of VSMCs into proliferative cells that could participate in stenosis and DCVS.

A previous study has shown that inhibiting the apoptosis pathway could significantly prevent the occurrence of DCVS [17], supporting the results of the present study. Indeed, the Akt pathway plays an important role in apoptosis, survival, proliferation, migration, metabolism, angiogenesis, and remodeling [19]. Akt is also involved in VSMC contraction [20] and in the balloon and stent damage model for the proliferation of VSMCs [21]. Phosphorylated Akt activates C-myc, which contributes to VSMC proliferation. Therefore, C-myc could be used as a marker of VSMC proliferation. In the present study, SAH increased p-Akt and C-myc expression, and these increases were partly prevented by cardamonin.

Studies have shown that cardamonin has anti-inflammatory, anti-viral, and anti-tumor effects [8, 9, 22-24]. At the same time, cardamonin can act as a vasodilator [11, 12] and is a potent antioxidant [25]. Liao et al. [11] have suggested that cardamonin may inhibit the mTOR pathway by insulin-induced thoracic aortic VSMCs proliferation. Nevertheless, there is no previous study on the effects of cardamonin on SAH-induced DCVS. The present study showed that cardamonin could act on p-AKT and C-myc and modulate the expression of α-SMA. These processes could help to prevent the occurrence of DCVS.

The present study suffers from some limitations. Indeed, no comprehensive study was performed on multiple markers of apoptosis and proliferation. In addition, the antioxidant and vasodilation effects of cardamonin were not assessed *in vivo*. Finally, even if the present study examined the effect of cardamonin on factors known to be associated with DCVS, the animals were not examined for the occurrence of DCVS, which could bias the results. In future studies, the animals might be followed and grouped according to the occurrence of vasospasm or not.

In conclusion, cardamonin seems to alleviate cerebral vasospasms after SAH. These effects may involve the inhibition of C-myc expression and apoptosis, and an increase of α-SMA expression. In addition, cardamonin also inhibits the Akt signal transduction pathway by decreasing protein kinase C.

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Disclosure of conflict of interest

None.

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