原　　著

Betulinic acid prevents lesion growth after venous ischemia in rats

KAORU HORIUCHI[1,2], ICHIRO NAKAGAWA[2], AXEL HEIMANN[1]
BEAT ALESSANDRI[1], HUIGE LI[3], OLIVER KEMPSKI[1]
Institute for Neurosurgical Pathophysiology, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany[1]
Department of Neurosurgery, Nara Medical University, Nara, Japan[2]
Department of Pharmacology, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany[3]

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Abstract

Background/Objectives : Betulinic acid is a component of a Chinese traditional herb that upregulates endothelial nitric oxide (NO) synthase (eNOS) and reduces NADPH expression. Increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) after cerebral arterial ischemia is a major cause of neuronal damage. Local venous ischemia can arise that slowly damages surrounding brain tissue during surgical procedures. The present study investigated the effects on infarct size when betulinic acid was administered after inducing two-vein occlusion (2VO) with a slowly developing lesion in rats.

Methods : We elicited 2VO in 18 male Wistar rats by the photochemical thrombosis of two adjacent cortical veins combined with KCL-induced cortical spreading depression (CSD). The rats were then randomized into groups (n = 9 each) to receive either dimethyl sulfoxide (DMSO) vehicle (control) or betulinic acid (30 mg/kg/day; n = 9) by daily gavage for seven days, and then infarct volume and 3-nitrotyrosine expression were assessed.

Results : Daily administration of betulinic acid for seven days significantly reduced infarct volume from 3.81 ± 0.7 to 1.90 ± 0.3 mm^3 ($p = 0.017$). Physiological data and regional cerebral blood flow did not significantly differ between the two groups during the study. We found 3-nitrotyrosine expression within the border zone of the infarct area and cleaved caspase-3 only within the lesion at the ipsilateral hemisphere in both groups. However, 3-nitrotyrosine/caspase-3 expression did not significantly differ at any time between the groups.

Conclusions : Betulinic acid reduced neuronal damage in a rat model of cerebral venous ischemia.

Key words : betulinic acid, penumbra, venous ischemia, nitrosative stress, oxidative stress

Introduction

Stroke is associated with high potential for mortality and disability, and ischemia/reperfusion induces neuronal damage that influences the outcomes of patients with ischemic stroke. Cerebral ischemia/reperfusion is associated with the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS)[1, 2]. The NADPH oxidase enzyme complex consists of membrane-bound cytochrome b558 and cytoplasmic proteins that translocate to the membrane during cellular stimulation to produce superoxide[3]; thus, NADPH oxidases are an important source of ROS. Nitric oxide (NO) derived via NO synthase (NOS) is thought to be beneficial for promoting collateral circulation and microvascular flow. Nitric oxide derived via neuronal NOS (nNOS) and inducible NOS (iNOS) is detrimental to the ischemic brain. In the normal brain, NO is non-toxic and functions as a second messenger. However, NO in the presence of superoxide anion radicals ($O_2^{\cdot}$-), reacts with $O_2^{\cdot}$- to form peroxynitrite (ONOO-) or nitrate (NO_3-), which damages the mitochondrial electron transport system, resulting in the impairment and death (apoptosis) of neuronal cells. Caspase-3, which is a potent effector of apoptosis that is triggered via several signaling pathways, might also play an important role in neuronal cell death caused by brain injuries including cerebral ischemia / reperfusion. Peroxynitrite generated via cerebral ischemia / reperfusion is highly cytotoxic and induces neuronal cell apoptosis mediated by caspase-3[4].

Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid) is a natural pentacyclic lupine triterpene with anti-cancer, anti-oxidant, anti-inflammatory, anti-HIV, and other effects found in Betula species of plants and various fruits and vegetables[5, 6, 7, 8]. It also upregulates eNOS and reduces NADPH oxidase expression in human endothelial cells[9]. A previous report showed that betulinic acid prophylaxis reduced infarct volume in a mouse model of middle cerebral artery (MCA) occlusion by reducing oxidative stress and nitrosative stress[10].However, whether betulinic acid confers therapeutic benefits in a later phase remains unknown. In contrast, cerebral venous infarction (CVI) can develop during neurosurgical procedures, such as brain retraction or those applied to treat venous injury, and cause slowly progressive ischemic damage to surrounding brain tissues. We have created a rat model of venous ischemia with concurrent widespread reduction of cortical cerebral blood flow (CBF) and a large ischemic penumbra[11, 12, 13, 14] that is appropriate for investigating penumbra pathophysiology[15]. Furthermore, when combined with induced cortical spreading depression (CSD), the effects on tissues are more damaging[16]. Here, we evaluated whether betulinic acid can reduce infarct growth in a rat model with venous ischemia induced using Rose Bengal and focused fiberoptic illumination. We also immunohistologically analyzed nitrotyrosine expression as an indicator of peroxynitrite-mediated, oxidative stress protein modification.

Methods

Animals

All experiments proceeded in accordance with German animal protection legislation.

Eighteen male Wistar rats (weight, 317 ± 29 g; Charles River Laboratories, Sulzfeld, Germany) were housed in individual cages and given free access to food and water. The rats were anesthetized with an intraperitoneal injection of chloral hydrate (36 mg/100 g body weight) before premedication with 1.0 mg atropine. Anesthesia was maintained with chloral hydrate (12 mg/100 g body weight/h) through a peritoneal polyethylene catheter. All animals were intubated with a silicon tube (outer diameter, 2.5 mm), mechanically ventilated with 30% O_2 using a Model 683 rodent ventilator (Harvard Apparatus, South Natick, MA, USA). We also assessed end tidal CO_2 using an Artema MM206C monitor (Heyer, Sundbyberg, Sweden). Rectal temperature was maintained at 37.0℃ using a feedback-controlled heating pad (Harvard Apparatus). A Portex polyethylene tube (Smiths Industries Medical Systems Co., London, UK) with an outer diameter of 0.80 mm was inserted into the tail artery to measure mean arterial blood pressure and gases using an ABL System 615 analyzer (Radiometer, Copenhagen, Denmark). Another polyethylene tube was inserted into the left femoral vein for intravenous Rose Bengal injection. The rats were mounted on a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA) and a 1.5 cm midline skin incision was created. A left parietal cranial window was prepared using a GD 604 high-speed drill (Aesculap, Tuttlingen, Germany) under an OP operating microscope (Zeiss, Wetzlar, Germany). During the craniotomy, the drill tip was continuously cooled with physiological saline to avoid thermal injury to underlying brain tissues. The dura mater was left intact.

Cortical vein occlusion by photochemical thrombosis

Two adjacent cortical veins with diameters of ~100 μm were occluded in rats with a similar venous anatomy via an intravenous (i.v.) injection of 50 mg/kg body weight of Rose Bengal (Sigma Chemical Co., St. Louis, MO, USA) and fiberoptic illumination using a 50-W mercury lamp (6500-7500 1 ×, 540 nm) and a 100-μm fiber[11, 12] (Fig. 1). After the first vein was occluded for 10-20 min from starting illumination, half of the initial Rose Bengal dose was injected i.v. into one vein, then the second vein was illuminated until occlusion. Venous occlusion was confirmed by laser Doppler imaging of acutely decreased blood flow at the occluded sites and surrounding tissues.

Measurement of CBF and tissue impedance

We measured CBF by laser Doppler flowmetry (Model BPM 403a; Vasamedics LLC, St. Paul, MN, USA) as described[11, 12], using a 0.8-mm needle probe at 25 points within the area between the occluded veins using a step-motor-driven and computer-controlled micromanipulator[12, 13]. Flow is expressed in LD units. Thus, one scan yielded information from 25 locations at 300- μm intervals. The CBF was scanned before, and every 5 min after venous ischemia for 75 min. The median observation frequency histogram correlated with absolute regional CBF (rCBF) determined by hydrogen clearance[17]. Two stainless steel impedance electrodes (outer diameter, 0.5 mm) were inserted into the cortex (depth, 0.4-0.5 mm; distance, 3 mm; Fig. 1) to measure cell swelling that occurs during induced CSD and venous ischemia[16]. The electrodes were covered with polyvinyl chloride for electrical insulation except at the 0.3-mm sharp-pointed tips. Impedance was measured at 1 kHz (10 mV, bias-free) throughout the study using a 4284A

precision LCR monitor (Hewlett-Packard Co., Avondale, PA, USA).

Induction of cortical spreading depression

As a part of the two-vein occlusion (2VO) model, 10 CSDs were elicited in all rats as follows. After inserting the impedance electrodes, a glass micropipette containing 150 mmol/L KCl was placed into the lateral cortex (Fig. 1) and linked to a CMA/100 microinjection pump (Carnegie Medicine, Stockholm, Sweden), then KCl was injected in 5.0- μ L volumes every 7 min to induce CSD[16, 18]. Injection started immediately after completing 2VO and finished 70 min thereafter (Fig. 2). The frequency of spontaneous CSD (sCSD) that occurred between the KCl-induced sCSD and the total number of CSDs correlated with infarction size.

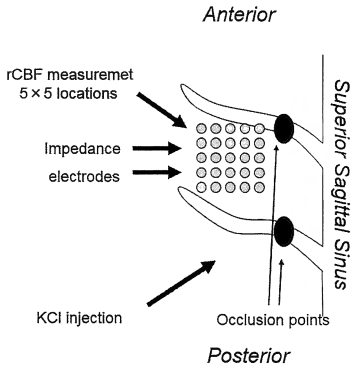

Fig.1.
Schema of experimental setup after craniotomy. Scan location for cerebral blood flow (25 locations), impedance electrodes, KCL microinjector, and occlusion points of two adjacent veins are shown. All instruments were placed in the parietal cortex to ensure that distances were comparable. rCBF, regional cerebral blood flow.

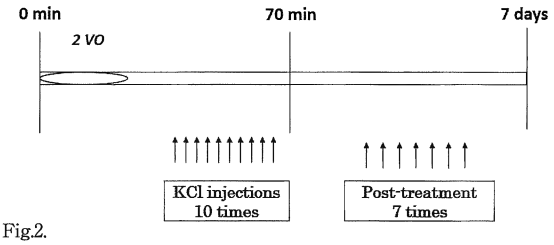

Fig.2.
Time course of drug administration, vein occlusion and KCL injection.

Experimental design and treatment groups

Rats were randomized into two groups (n = 9 each) and administered via gavage with either dimethyl sulfoxide (DMSO) vehicle (control) or betulinic acid (Sigma-Aldrich, St. Louis, MO, USA) dissolved in DMSO (final concentration, 10 mg/mL) in doses of 30 mg/kg/day for seven days from 90 minutes after 2VO.

Histological determination of infarct volume

All rats were perfusion-fixed with 4% paraformaldehyde under deep anesthesia on day 7 after 2VO. Brains were removed from the skull, embedded in paraffin, then the parietal region including the infarct area was sliced into 3-μm-thick coronal sections and stained with hematoxylin and eosin. Infarct volume was histologically evaluated using a light microscope connected to a charge coupled device (CCD) camera (Sony, Tokyo, Japan) and Optimas 6.51 software (Optimas Corp., Seattle, WA, USA). The infarct area was evaluated in 200-μm steps on serial sections. Infarction volume was calculated from the sum of all measured lesion areas (mm^2) multiplied by the distance between sections (0.2 mm).

Immunohistological analysis of 3-nitrotyrosine/caspase-3

Nitrotyrosine indicates cell damage, inflammation and the production of intracellular

NO. Therefore, nitrotyrosine concentrations were determined by immunohistochemistry. Deparaffinized and rehydrated brain sections were shaken overnight with polyclonal rabbit-anti-nitrotyrosine primary antibody (5 μg/mL; 1:500; Millipore, Cat.# 06-284). Rinsed sections were covered in biotinylated secondary antibodies (Vectastain ABC Kit PK-6101, Rabbit IgG) for one hour in a humid chamber, followed by two minutes in peroxidase solution and DAB (Vectastain ABC Kit PK-6101, Rabbit IgG). Two different individuals counted cells that were immunohistologically positive for nitrotyrosine three times in a blinded manner in slices classified into three categories. Values were then averaged and summarized.

Statistical analysis

Data were compared using one-way analyses of variance and results are expressed as means ± standard error of the mean. Statistical significance was assumed at $p < 0.05$ (Sigmastat 3.1, Systat Software Inc. Chicago, IL, USA).

Results

Physiological data

Physiological parameters did not significantly differ, and blood gases including PaO_2 and $PaCO_2$ were within normal ranges in both groups throughout the present study. Mean arterial pressure and rectal temperature did not significantly change after 2VO, and body weight did not significantly differ between the groups on days 0 and 7 (Table 1).

Table 1. Physiological variables before and after venous ischemia.

			Blood gas analysis		Weight(g)	
	MABP(mmHg)	Rectal temperature(℃)	PaO_2(mmHg)	$PaCO_2$(mmHg)	day0	day7
Vehicle						
Before 2VO	90.3±4.7	37.2±0.1	39.01±1.1	123.7±4.0	301.5±10.6	257±15.8
After 2VO	87.5±2.3	37.4±0.1	39.8±1.0	122.8±3.9		
Betulinic Acid						
Before 2VO	88.4±4.3	37.4±0.1	38.7±1.1	118.8±4.5	324±8.3	285.4±11.5
After 2VO	85.8±3.7	37.3±0.1	37.1±1.8	124.2±4.6		

Vehicle=DMSO; MABP=mean arterial blood pressure; PaO2=partial pressure of oxygen; PaCo2=partial pressure of carbon dioxide; 2VO=two vein occlusion. The data are expressed as mean ± standard error of mean (SEM).

Changes in rCBF

Median rCBF values from the 25 locations in each animal did not significantly differ between the control and betulinic acid groups (Fig. 3) during the comparative phase before ischemia (50.0 ± 6.2 vs. 42.3 ± 6.8 LD units). These values were reduced to 22.8 ± 2.3 vs. 17.9 ± 2.1 LD units in the respective groups at 70 min after 2VO (no significant difference; Fig. 3).

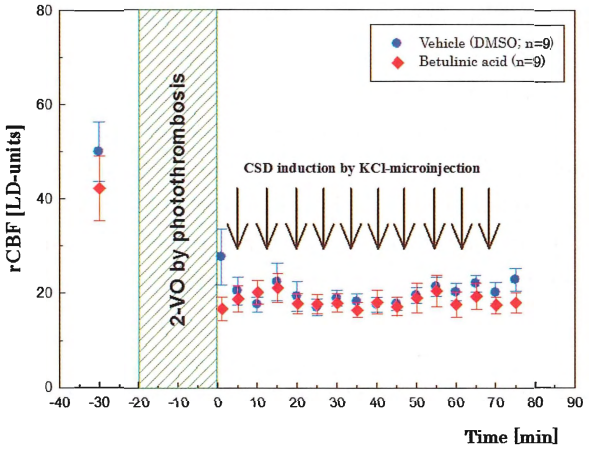

Fig.3.
Sequential changes in regional CBF. Median local CBF data from individual rats show ~ 50% reduction in rCBF after venous occlusion in both groups without significant differences. CSD: cortical spreading depression.

Changes in tissue impedance and the number of CSD

Impedance and spontaneous CSD occurrence were measured in both groups. The total numbers of induced + spontaneous CSD did not significantly differ between the groups during the initial 75 minutes after 2VO (Fig. 4).

Infarct volume

All the rats developed cortical infarcts. However, the infarct volume at seven days after 2VO was significantly reduced in the group given betulinic acid compared with the control group (1.90 ± 0.3 vs. 3.81 ± 0.7 mm^3, $p = 0.017$; Fig. 5).

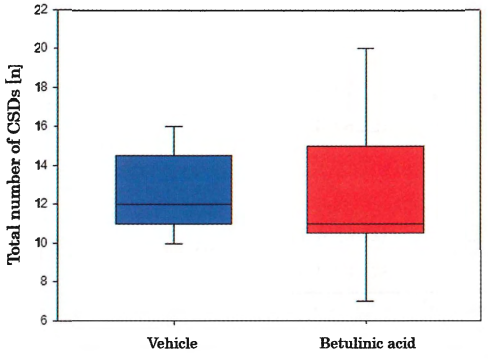

Fig.4.
Numbers of induced and spontaneous CSDs in experimental and control groups during first 75 min after 2VO. Values do not significantly differ between groups. CSD: cortical spreading depression.

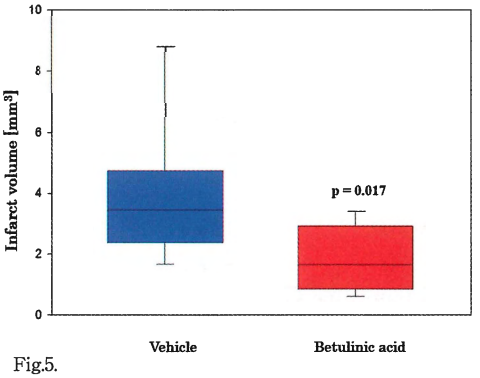

Fig.5.
Infarct volumes (mm^3) at seven days after occlusion of two veins. Values are shown as means ± standard error of mean (SEM). Infarct volume is reduced in the group given betulinic acid compared with the control. *$p = 0.017$. DMSO: dimethyl sulfoxide.

Expression of 3-nitrotyrosine/caspase-3

Positive 3-nitrotyrosine staining within the border zone of the infarct area and cleaved caspase-3 only within the lesion at the ipsilateral hemisphere were detected in both groups (Fig. 6). However, these values did not significantly differ between the groups at seven days after 2VO (data not shown).

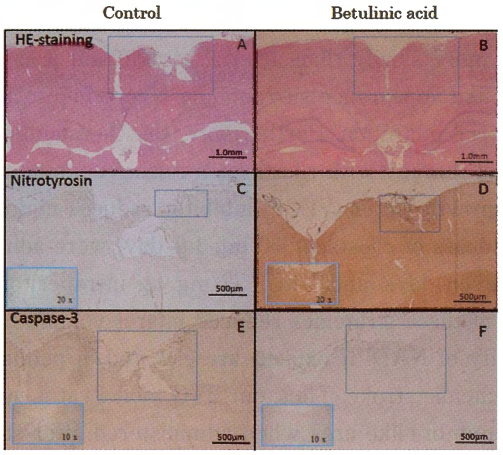

Fig.6.
Findings of hematoxylin and eosin staining and 3-nitrotyrosine/caspase-3 immunolabeling. Typical findings of HE staining (A, B), nitrotyrosine (C, D) and cleaved caspase-3 (E, F). Infarctions were smaller in rats treated with betulinic acid (B) than in the control (A). Infarct area and border zone are 3-nitrotyrosine-positive (C, D). Isolated, cleaved caspase-3 is evident inside the lesion in the ipsilateral hemisphere (E, F). Positive cells on day 7 after 2VO do not significantly differ between the control (C, E) and betulinic acid groups (D, F). Original magnification × 2.5 (A, B) and × 5.0 (C–F). Bar, 1 mm (A, B) and 500μm (C–F). HE, hematoxylin-eosin.

Discussion

We showed that betulinic acid reduced infarct volume in a rat model of venous ischemia, although 3-nitrotyrosine/caspase-3 values did not significantly differ between the groups at seven days after 2VO.

Cerebral ischemia and reperfusion increase ROS formation[19] and NADPH oxidases are a major source of ROS in cerebral ischemia-reperfusion injury[11, 20, 22], as they mediate blood-brain barrier (BBB) disruption and contribute to tissue damage[21]. Therefore, inhibiting NADPH oxidase expression and/or activity might serve as a novel therapeutic strategy for stroke. The classic NADPH oxidase inhibitors, diphenyleneiodonium and apocynin, are non-specific and cannot be used as drugs[23], but the plant-derived pentacyclic triterpenes, ursolic acid and betulinic acid, reduce vascular NADPH oxidase expression in human endothelial cells[8, 24]. Therefore, these agents might be able to reduce neuronal injury after stroke. Prophylactic betulinic acid (50 mg/kg/day) for seven days reduced infarct volumes 22 h after reperfusion in model mice with MCA occlusion by reducing oxidative and nitrosative stress[10]. However, whether betulinic acid confers therapeutic benefits in a later phase remains unknown. Furthermore, CVI can develop during neurosurgical procedures, such as brain retraction or those applied to treat venous injury, and cause slowly progressive ischemic damage to surrounding brain tissues. Whether betulinic acid confers neuroprotective effects when administered

after CVI remains unknown. Therefore, we investigated whether betulinic acid could reduce established infarct growth in a rat model of venous occlusion.

The occlusion of two cortical veins is associated with a rather widespread area of reduced CBF[12] and flow is hardly detectable during the acute phase[16]. Thus, this model of venous ischemia has been proposed as a penumbra model[12, 15, 16]. Small infarcts develop within 24 hours in the 2VO model then continuously grow for at least four days[13, 25, 26, 27, 28, 29]. Therefore, CVI generally develops much more slowly than arterial stroke, and areas of moderately reduced CBF causing a penumbra-like appearance surrounding a core infarct in CVI are wider than in those associated with arterial infarction. Furthermore, the therapeutic window is much wider in CVI, and post-procedural therapy with cilostazol, a selective inhibitor of phosphodiesterase-3, and vascular endothelial growth factor (VEGF) inhibitor reduces neuronal damage in our 2VO model rats[28, 30]. Daily oral doses of cilostazol (60 mg/kg/day) were administered one hour after 2VO and continued for seven days, and VEGF (2 mg/kg intraperitonially) was administered 24 hours after 2VO. Both of these strategies reduced infarct volume by seven days after 2VO. The expression and activity of NADPH oxidase are elevated in penumbral arteries[31, 32] and in neuronal tissues[33] after ischemic stroke. Thus, our 2VO model rat is suitable for evaluating the effects of drugs on the penumbra-like area when administered after stroke. The present study found that the post-procedural administration of betulinic acid in the rat 2VO model resulted in a neuroprotective effect upon CVI, suggesting that betulinic acid has therapeutic potential for treating venous injury or occlusion during surgical interventions, if administered during the acute phase. Moreover, betulinic acid is safe and non-toxic in mice even at doses up to 500 mg/kg body weight[34]. Thus, high doses of betulinic acid could be administered during the acute phase of cerebral arterial occlusion.

Pharmacological and genetic approaches have significantly advanced understanding of the roles that NO and NOS isoforms play in focal cerebral ischemia. Both nNOS and iNOS play key roles in neurodegeneration, whereas eNOS plays a prominent role in maintaining CBF and preventing neuronal injury[35]. Betulinic acid enhances eNOS expression in cultured endothelial cells[9] and increases eNOS expression while reducing nNOS and iNOS expression in mouse models of MCA occlusion[10]. Peroxynitrite generation is a crucial pathogenic mechanism under conditions of myocardial infarction, chronic heart failure, diabetes, circulatory shock, chronic inflammatory diseases, cancer and neurodegenerative disorders[36, 37]. Nitric oxide is non-toxic and functions as a second messenger in the normal brain, but reacts with $O_2^{\cdot}$- to form ONOO- or NO_3 in the presence of $O_2^{\cdot}$-, thus damaging the mitochondrial electron transport system and neurons. Peroxynitrite-derived radicals cause the direct nitration of tyrosine residues. Proteins containing nitrotyrosine residues have been detected in several pathological conditions associated with enhanced oxidative stress[38]. Therefore, 3-nitrotyrosine could serve as an indicator of peroxynitrite formation and oxidative stress[39]. We postulated that betulinic acid would reduce lesion growth after 2VO by preventing peroxynitrite radical production. We detected 3-nitrotyrosine within, and at the border zone of infarcts in both groups, but nitrotyrosine values did not significantly differ between them at seven days after 2VO. Values for 3-nitrotyrosine peak within the first 48 hours, then gradually decrease in mouse models of traumatic brain injury[40]. The peak 3-nitrotyrosine value is maintained until 12 h after injury,

and returns to baseline at 24 hours[41]. The time-course of 3-nitrotyrosine level might be the same in models of venous ischemia. Therefore, we speculated that these values similarly leveled out in both groups by seven days after 2VO in the present study, even though small infarcts continued to grow for at least four days.

Apoptosis occurred sequentially and widely in cortical lesions considered as penumbra-like areas in our rat 2VO model[42]. Thus, we consider that the control of apoptotic changes plays an important role in neuroprotection against venous ischemia. Betulinic acid exerts anti-apoptotic effects against isoflurane-induced brain damage in newborn rats[43] and upregulates p-PI3K and p-Akt expression in hippocampal neurons induced by glucose deprivation/reperfusion[44], and Akt exerts anti-apoptotic effects through inactivation of pro-apoptotic proteins such as Bad and caspase-9[45]. Caspase-3 is a potent effector of apoptosis triggered via several signaling pathways, and it might play important roles in neuronal cell death resulting from various brain injuries. We detected caspase-3 within lesions at the ipsilateral hemisphere seven days after 2VO in both groups. Values for 3-nitrotyrosine might normalize by seven days after 2VO, because peroxynitrite generated by cerebral ischemia/reperfusion induces apoptotic neuronal cell death mediated by caspase-3[4]. Therefore, caspase-3 expression did not differ between the two groups. We speculate that these anti-apoptotic effects of betulinic acid could contribute to the reduction of neuronal damage in the rat 2VO model.

The present study has some limitations. We examined the effects of betulinic acid at seven days after venous ischemia on infarct size in a rat 2VO model. Although CVI generally develops much more slowly in this model, 3-nitrotyrosine/caspase-3 values might have already been attenuated by seven days after 2VO. We could not identify any significant differences in 3-nitrotyrosine/caspase-3 expression between the groups. Earlier time points should be immunohistologically investigated.

In conclusion, we showed that betulinic acid minimized venous ischemic injury in the rat 2VO model. These findings suggest that betulinic acid has therapeutic potential for treating cerebral venous ischemia.

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