Calpeptin Reduces Neurobehavioral Deficits and Neuronal Apoptosis Following Subarachnoid Hemorrhage in Rats

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Background: Inhibition of calpain activity provides neuroprotection in multiple central nervous system injury, but the role and mechanism of calpain in subarachnoid hemorrhage (SAH) remain unclear. This study was undertaken to determine the effects of inhibition of calpain on neurological deficit and neuronal apoptosis following experimental SAH. *Methods:* The endovascular perforation model of SAH was produced in male Sprague-Dawley rats. Rats were administered calpeptin 50 μg, intracerebroventricular injection, 30 minutes before induction of SAH. After 72 hours, the method of Evans blue dye extravasation and wet/dry method were used for determination of blood-brain barrier permeability and brain edema, Western blot analysis and immunohistological staining were used to evaluate neuronal apoptosis. *Results:* The intracellular Ca^{2+} level and calpain activity was significantly elevated in basal cortex after SAH. Calpain inhibitor calpeptin reduces brain water content and Evans blue dye extravasation, improves neurobehavioral deficits after SAH. Importantly, calpeptin treatment significantly reduces activation of caspase-3, caspase-9, caspase-12 and poly ADP ribose polymerase and the number of apoptotic neurons in basal cortex after SAH. *Conclusion:* The present study suggested that calpeptin is neuroprotective in early brain injury after SAH through antiapoptotic effect.

Key Words: Subarachnoid hemorrhage—calpain activity—calpeptin—neuronal apoptosis

Introduction

Subarachnoid hemorrhage (SAH) is a fatal subtype of stroke in which blood flows into subarachnoid space in the brain and which is often caused by aneurysm rupture. Directly after SAH, the arterial blood pressure and intracranial pressure increase, the cerebral blood flow and cerebral perfusion pressure and oxygen tension decrease, ionic distribution is rapidly impaired and promotes disturbance in electrical activity, these pathological changes cause the initiation of cerebral inflammation and cell apoptosis.[1,2] Disruption of intracellular calcium homeostasis is a key factor in the pathophysiology of SAH. There is a massive calcium influx secondary to glutamate release and glutamate receptor activation-mediated calcium release after SAH. Experimental studies show that a pathological rise in intracellular calcium in neurons, endothelial, and smooth muscle cells after SAH.[3-6] Excessive intracellular calcium accumulation causes neuronal degeneration through induction of free radical release, activation of phosphatases and phospholipases, and mediating detrimental changes of gene expression.[7,8]

Calpain is a Ca^{2+}-dependent neutral cysteine hydrolase, widely expressed in the cytoplasm of mammalian tissue and plasma membrane, and has function of degrading cytoskeletal protein and signaling enzyme.[9] Calpain played an important role in neuronal death and neurodegeneration.[10,11] Previous studies showed that calpain-mediated spectrin breakdown products levels of cerebrospinal fluid are significantly increased in aneurysmal SAH patients,[12,13] suggest that calpain maybe play an important role in process of brain injury following SAH. Systemic administration of a calpain Inhibitor II reduces

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behavioral deficits and blood−brain barrier in experimental SAH.[14] However, it is not known whether calpain affects neuronal apoptosis after SAH. To explore the possible role of calpain in SAH, we utilized a synthetic calpain inhibitor, calpeptin in the present study, which has high efficiency, potency, and specificity toward calpain.[15]

In the present study, we hypothesized calpeptin may alleviate neuronal apoptosis via inhibition of calpain in early brain injury after experimental SAH.

Materials and Methods

Experimental Animals

The experimental protocols and ethical guideline were approved by the Animal Use and Care Committee at the Xiangyang Central Hospital. In the present study, the experiment consisted of 2 parts. (1) Thirty-two male Sprague-Dawley rats (Experimental Animal Center of Hubei University of Arts and Science), weighing between 280 and 330 g, were randomly divided into the sham group and SAH group on day 1, 2, and 3 (n = 8 for each group). The brain tissue was taken at the above time point for measuring intracellular Ca^{2+} concentration and calpain activity (n = 6). (2) Seventy rats were divided into the sham group (n = 18), SAH + vehicle group (n = 26), and SAH + calpeptin group (n = 26). Eight rats per group were randomly assessed for neurobehavioral tests. Six rats in each group were killed. The basal cortex in the left hemisphere was collected and used for Western blot analysis. The remaining left hemisphere, right hemisphere, cerebellum, and brain stem were used for brain water content. Six rats in each group were used for detecting blood-brain barrier (BBB). Six rats in each group were for used for immunohistological staining.

Induction of SAH and Grading System

Rat SAH model was produced by endovascular perforation method as previously described.[16] Briefly, after exposing the external carotid artery (ECA), the left common carotid artery and the internal carotid artery (ICA), the ECA was ligated. A sharpened 4-0 nylon suture was advanced into the intracranial ICA from the ECA until resistance was felt (about 18 mm from the common carotid bifurcation) and then pushed 3 mm to perforate the wall of ICA. The suture was quickly withdrawn and produced SAH model. The sham group underwent identical procedure except no perforation. SAH grade was performed by using a grading system. [16] Briefly, the basal cistern of rat was divided into 6 segments, each segment was scored from grade 0 to grade 3 depending on the subarachnoid blood clot as follows: grade 0 (no subarachnoid blood); grade 1 (minimal subarachnoid blood); grade 2 (moderate subarachnoid blood clot with arteries); grade 3 (subarachnoid blood clot obliterating all arteries). The SAH grade was in a total score of 6 segments.

Drug Injection

In calpeptin-treated SAH group, rat received a single intracerebroventricular injection of calpeptin (50 μg in 5 μL DMSO; Tocris, #0448, UK) or vehicle before the left internal carotid artery perforation for 30 minutes. Dosage and time of calpeptin administration was based upon prior investigation in focal cerebral ischemia model,[17] the intracerebroventricle injection was performed in SAH model as previously reported.[18]

Neurobehavioral Tests

The behavioral deficits were assessed at 72 hours after SAH using the rotarod test, and beam balance test as previously described.[19] Briefly, the rotarod test was performed using the accelerating rotarod test (Ugo Basile), rats of each group was trained 3 trials each day for 5 days before SAH, and trained to stay on an accelerating rotarod (4-40 rpm over 5 minutes, with an increasing stem of 4 rpm every 30 second interval). The mean fall latency of each group was recorded. The beam balance test was performed on a 60 cm square narrow wooden beam (1 cm wide and 50 cm above from the floor). The duration the rat of each group remained on the center of the beam was recorded, with a maximum of 60 seconds.

The neurological deficit was evaluated at 72 hours after SAH with a modified Garcia scoring system.[16] Briefly, spontaneous activity, spontaneous movements of all limbs, movements of forelimbs, climbing wall of wire cage, reaction to touch on both side of trunk, and response to vibrissae touch were tested by an observer blinded to the experiment (0-18 scores).

Measurement of Intracellular Ca^{2+} and Calpain Activity

Rats of each group were sacrificed under anesthesia after SAH and the cerebral cortex was harvested. Intracellular Ca^{2+} concentration and calpain activity were conducted with a calcium assay kit (ab102505, Abcam) and a calpain activity assay kit (ab65308, Abcam) according to the given protocol from the manufacturer.

Western Blot Analysis

Rats of each group were sacrificed under anesthesia after SAH and the cerebral cortex was harvested. The cerebral cortex was prepared by homogenization in radioimmunoprecipitation assay lysis buffer (P0013, Beyotime, China) and then determined the protein concentration with Bradford protein assay kit (P0006, Beyotime, China). The equal extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to the nitrocellulose membrane. Then blots were blocked with nonfat milk (5 %) and then incubated with primary antibodies: (anti-calpain 1, ab28258; anti-calpain 2, ab39165; anti-caspase-12, ab62484, Abcam), (anti-caspase-3, #9662; anti-caspase-9, #9508; poly ADP ribose polymerase [PARP], #9542,

Cell Signaling Technology), and (anti-spectrin, sc-48382; anti-β-actin, sc-47778; anti-ZO-1, sc-8147; anti-occludin, sc-8144; anti-claudin-5, sc-28670, Santa Cruz Biotechnology) overnight at 4°C. The membranes were washed thrice in tris-buffered saline with tween 20 buffer (P0023, Beyotime, China), and incubated with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase for 2 hours. The membranes were washed thrice in tris-buffered saline with tween 20 buffer. Then protein band was visualized by chemiluminescence kit (#20148, ThermoFisher) and exposure to film.

Brain Water Content and Evans Blue Assay

Brain water content was quantified by the wet/dry method after SAH as previously described.[18] Rats of each group were sacrificed under anesthesia after SAH and the brains were harvested. The brains were quickly separated into the right hemisphere, left hemisphere, cerebellum, and brain stem. Specimens were immediately weighed to determine the wet weight. The dry weight was quantified after dried at 100 °C.

The BBB permeability was evaluated by Evans blue dye extravasation according to previous study.[20] Briefly, Evans blue (2% in saline, 3 mL/kg) was injected intravenously and was allowed to circulate for 1 hour. Rat was perfused transcardially with saline. The harvested brains were quickly separated into the right hemisphere and left hemisphere, and determined the weight. Brain samples were homogenized in 2 mL phosphate buffer saline (PBS) buffer, mixed with 2 mL of 50 % trichloroacetic acid, and centrifuged for 10 minutes. The supernatant was measured at 620 nm (absorbance of Evans blue) using a spectrophotometer (Molecular Devices, SpectraMax M5e). The content of Evans blue was represented as μg/g against a standard curve.

Immunohistological Staining

The double label staining of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) with neuronal nuclei (NeuN) was performed to assess neuronal apoptosis in basal cortex as previously described.[21] Rats of each group were perfused transcardially with 4% paraformaldehyde/PBS under anesthesia after SAH. The brains was taken and immersed in 4% paraformaldehyde/20% sucrose solution overnight. The 10 μm thick-coronal sections (at bregma +0 to 3 mm) were blocked with 5% goat serum containing 0.1% Triton X-100 for 30 minutes, and then incubated with NeuN (Neuronal Marker, 1:500, ab104224, Abcam) overnight at 4°C. The coronal sections washed with PBS buffer and then incubated with anti-mouse IgG (Alexa Fluor 594, ab150116, Abcam). Next, the sections were used for TUNEL (TUNEL, QIA39, Merck, Germany). The stained sections were viewed with a confocal microscope (Leica SP5, Germany). The number of TUNEL/NeuN positive cells was quantified and averaged as per mm^2 (6 random images) in basal cortex.

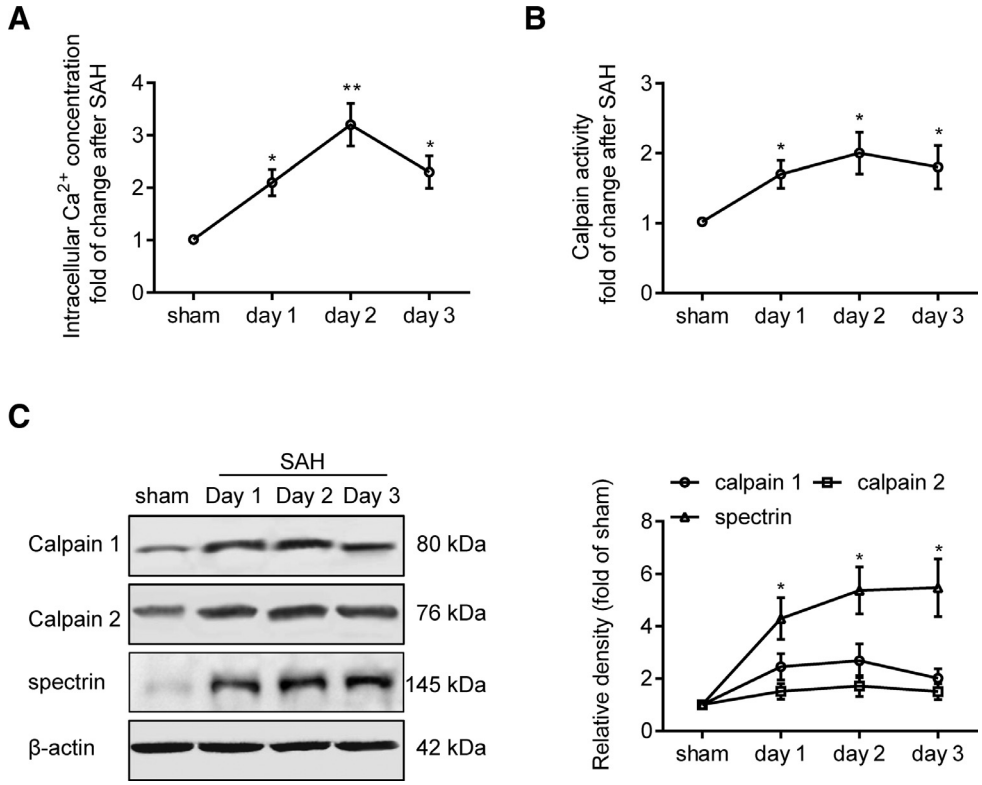

Figure 1. ***Calpain activity increased after SAH****. (A) The levels of intracellular Ca^{2+} concentration, (B) calpain activity, (C) levels of calpain 1, calpain 2, and spectrin, were analyzed in rat brain basal cortex in the sham and SAH groups at day 1, day 2, and day 3 (n = 6, P < 0.05 and P < 0.01 versus sham).*

Statistical Analyses

Value are represented as the mean ± SEM (standard error of mean), and analyzed with one-way ANOVA that was provided by the computer program SPSS 13.0 statistical software. *P* less than .05 was considered statistically significant.

Results

SAH Significantly Increased Calpain Activity

Rat SAH model was produced by endovascular perforation method. We dissected the basal cortex for biochemical analysis at various time points. Compared to the sham group, the level of intracellular Ca^{2+} concentration and calpain activity were significantly increased in the basal cortex at day 1, day 2, and day 3 following SAH (Fig 1A and B). To confirm the calpain activation after SAH, we determined the protein expression of calpain 1, calpain 2, and the level of spectrin degradation fragment. Western blot analysis showed that compare to the sham group, the level of calpain 1 and calpain 2 increased, the 145 kDa spectrin degradation band that is used as a marker of calpain activation, significantly increased in the basal cortex at day 1, day 2, and day 3 after SAH (Fig 1C). The above results suggested that SAH increased calpain activity.

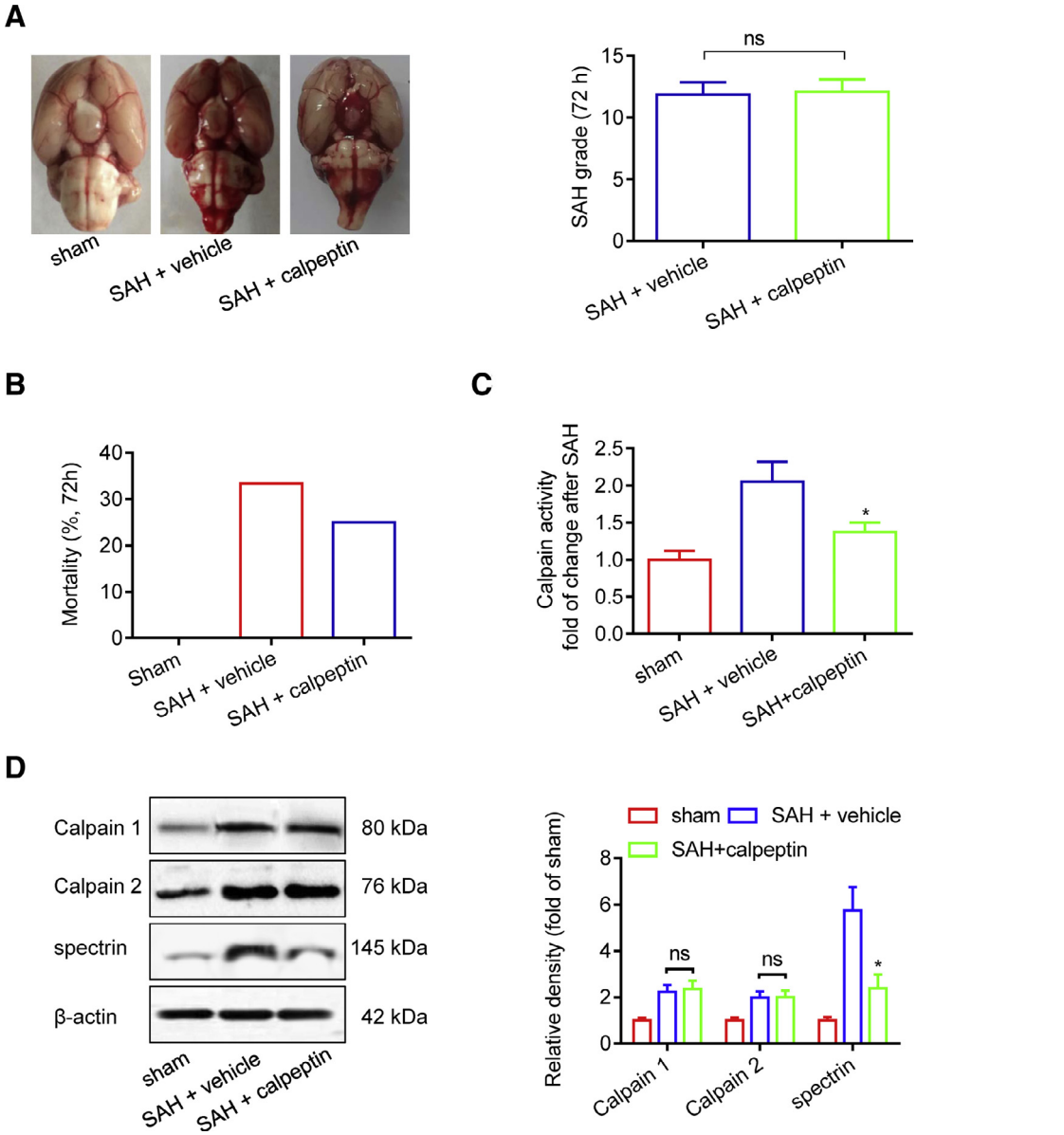

Figure 2. ***Calpeptin attenuated SAH-elevated calpain activity at day 3****. (A) Representative images showing the rat brain for sham group, vehicle or calpeptin-treated SAH group. The SAH grade showed no significant difference between the SAH + vehicle group and the SAH + calpeptin group (n = 12). (B) The mortality rate, (C) calpain activity, (D) levels of calpain 1, calpain 2, and spectrin were analyzed in sham group, vehicle or calpeptin-treated SAH group (n = 6, P < 0.05 versus SAH + vehicle group; ns, no significant).*

Calpeptin Attenuated SAH-Elevated Calpain Activity

The SAH grading scores were 11.8 ± 1.1 and 12.1 ± 0.9 in SAH + vehicle group and SAH + calpeptin group, respectively. There was no significant difference between 2 groups (Fig 2A). The SAH + vehicle group mortality rate was 26.9 % (7 of 26 mice), and the SAH + calpeptin group rate was 23.1 % (6 of 26 mice; Fig 2B). Results showed that calpeptin significantly attenuated SAH-elevated calpain activity (Fig 2C). Moreover, calpeptin treatment did not alter the level of calpain 1 and calpain 2, while reduced SAH-elevated spectrin degradation band (Fig 2D), suggesting that injection of calpeptin could inhibit the calpain activity after SAH.

Effect of Calpeptin on Body Weight and Neurobehavior Assessment After SAH

The difference in body weight on day 1 and day 2 among the sham, SAH + vehicle group and SAH + calpeptin group was not significant. Calpeptin treatment significantly restored the body weight on 3 days after SAH, as compared to the SAH + vehicle group (Fig 3A). Compared to the sham group, SAH caused impairment in the ability to remain on the rotarod and beam at day 3, while calpeptin-treated SAH group exhibited significantly improved time of rotarod and beam balance (Fig 3B and C). Moreover, the modified Garcia score significantly worse at day 3 in SAH + vehicle group compared to sham group. However, the neurological deficits had improved with calpeptin treatment compared with SAH + vehicle group (Fig 3D). Taken together, the above results indicated that calpeptin treatment significantly improved SAH-induced neurobehavior deficit.

Effect of Calpeptin on Brain Edema and Blood-Brain Barrier Disruption After SAH

An increase in brain water content was observed in SAH group at 72 hours compared with sham group, while calpeptin treatment significantly reduced SAH-elevated brain water content in left and right hemisphere compared to vehicle group (Fig 4A). Second, the SAH + vehicle group showed obvious extravasation of Evans blue dye into the left and right hemisphere at day 3. However, the amount of Evans blue extravasation was significantly reduced in the SAH + calpeptin group, when compared to the SAH + vehicle group (Fig 4B). Moreover, expression of tight junction protein claudin-5, occludin, and ZO-1 decreased in the SAH + vehicle group at day 3, when compared with that of sham group, while calpeptin could restore SAH-induced decrease (Fig 4C). These results indicate that calpeptin treatment significantly attenuated brain edema and BBB disruption after SAH.

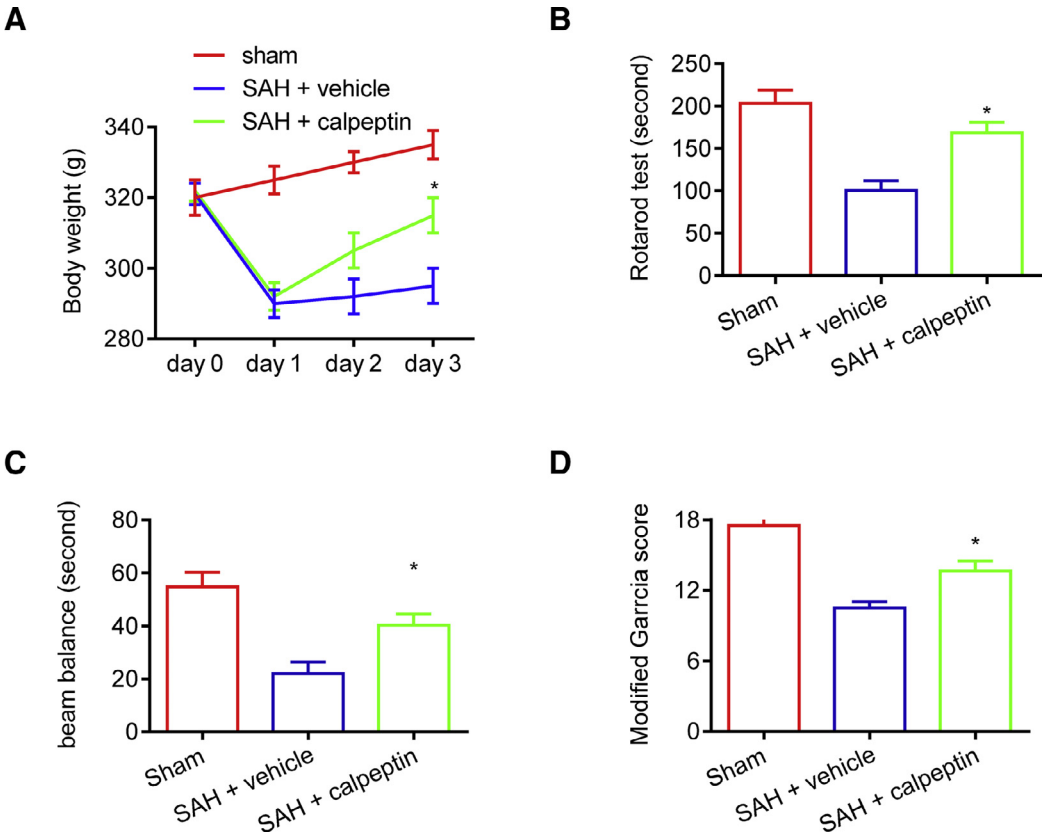

Figure 3. ***Calpeptin improved SAH-induced neurobehavioral deficits at day 3****. (A) Body weight, (B) rotarod test, (C) beam balance test, and (D) modified Garcia score were analyzed in sham group, vehicle or calpeptin-treated SAH group (n = 8, P < 0.05 versus SAH + vehicle group).*

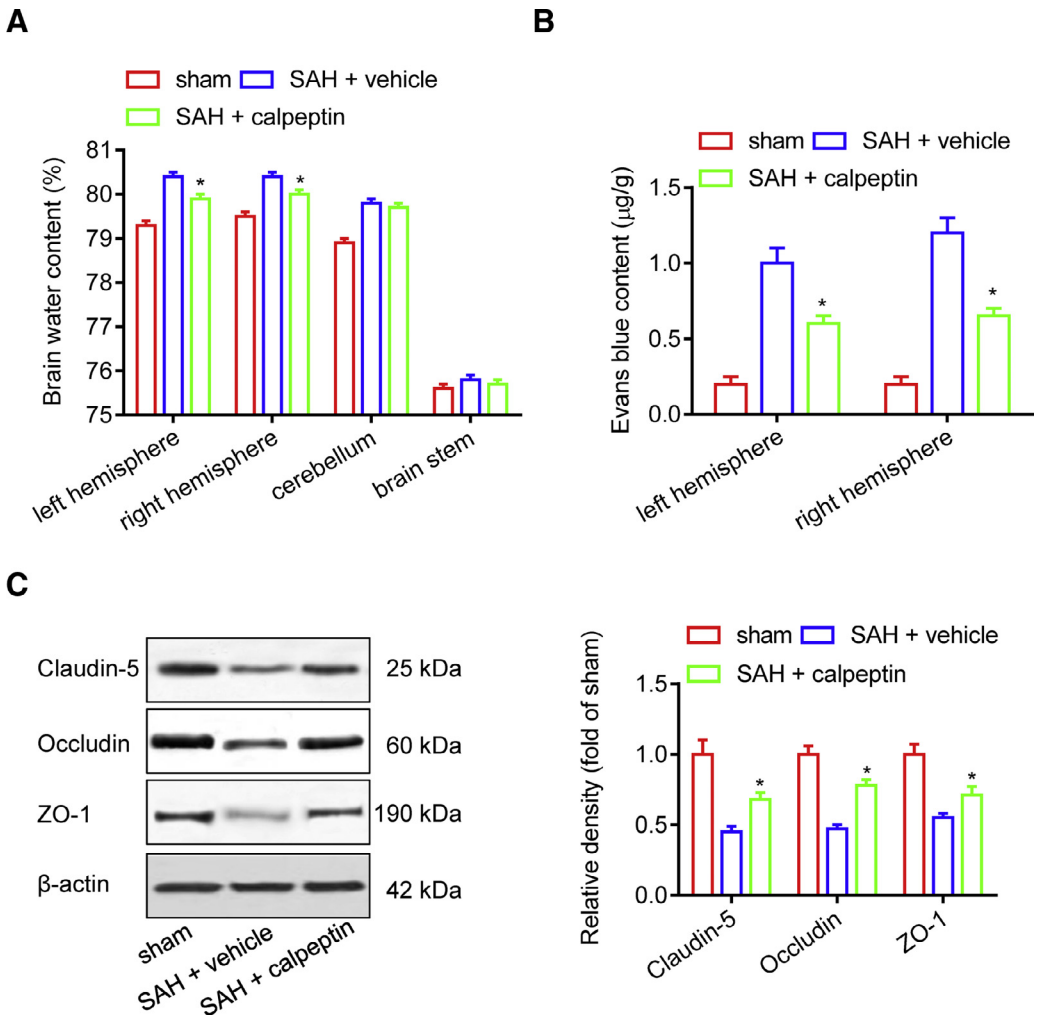

Figure 4. ***Calpeptin attenuated SAH-induced brain edema and BBB disruption at day 3***. *(A) Brain water content, (B) Evans blue dye extravasation, (C) levels of ZO-1, Occludin and claudin-5 were analyzed in sham group, vehicle or calpeptin-treated SAH group (n = 6, P < 0.05 versus SAH + vehicle group).*

Effect of Calpeptin on Neuronal Apoptosis After SAH

We assessed the cellular apoptosis of basal cortex at day 3 by using the Western blot analysis and TUNEL/NeuN immunofluorescence staining. As shown in Fig 5, cleaved caspase-3, cleaved caspase-9, cleaved caspase-12, and cleaved PARP were significantly increased in the SAH + vehicle group as compared to the sham group, while calpeptin treatment could reduce SAH-induced the caspase activation. Statistics results showed that TUNEL positive neurons obviously increased in basal cortex of the SAH + vehicle group when compared to the sham group, which is inhibited by calpeptin treatment (Fig 5B). These findings indicated that calpeptin treatment significantly attenuated neuronal apoptosis after SAH.

Discussion

In this study, we show that intracerebroventricle injection of calpeptin attenuated brain edema and BBB disruption, activation of caspase-3, caspase-9, caspase-12, and PARP, neuronal apoptosis, and improved neurobehavioral deficits after SAH. These results suggest that calpeptin is neuroprotective in early brain injury after SAH through an antiapoptotic mechanism.

Calpain is a family of calcium-activated proteolytic enzymes, calpain 1 and calpain 2 are the 2 isoforms ubiquitously distributed in the central nervous system. Calpain is important for neural development and degeneration and synaptic plasticity, while has deleterious effect on neuron upon their pathological over-activation.[10,11] Subarachnoid hemorrhage will lead to the overload of intracellular Ca^{2+},[2] then Ca^{2+} binds the catalytic domain of calpain to cause its activation. As observed in this study, our results showed that SAH obviously caused the increase of intracellular Ca^{2+} and activation of calpain. The activated calpain degrades a variety of key cytoskeletal proteins, lysosomal membrane protein, calcium-dependent transcription factor, and caspase family members, caused the lysosomal rupture and the mitochondrial permeability transition finally leading to cell

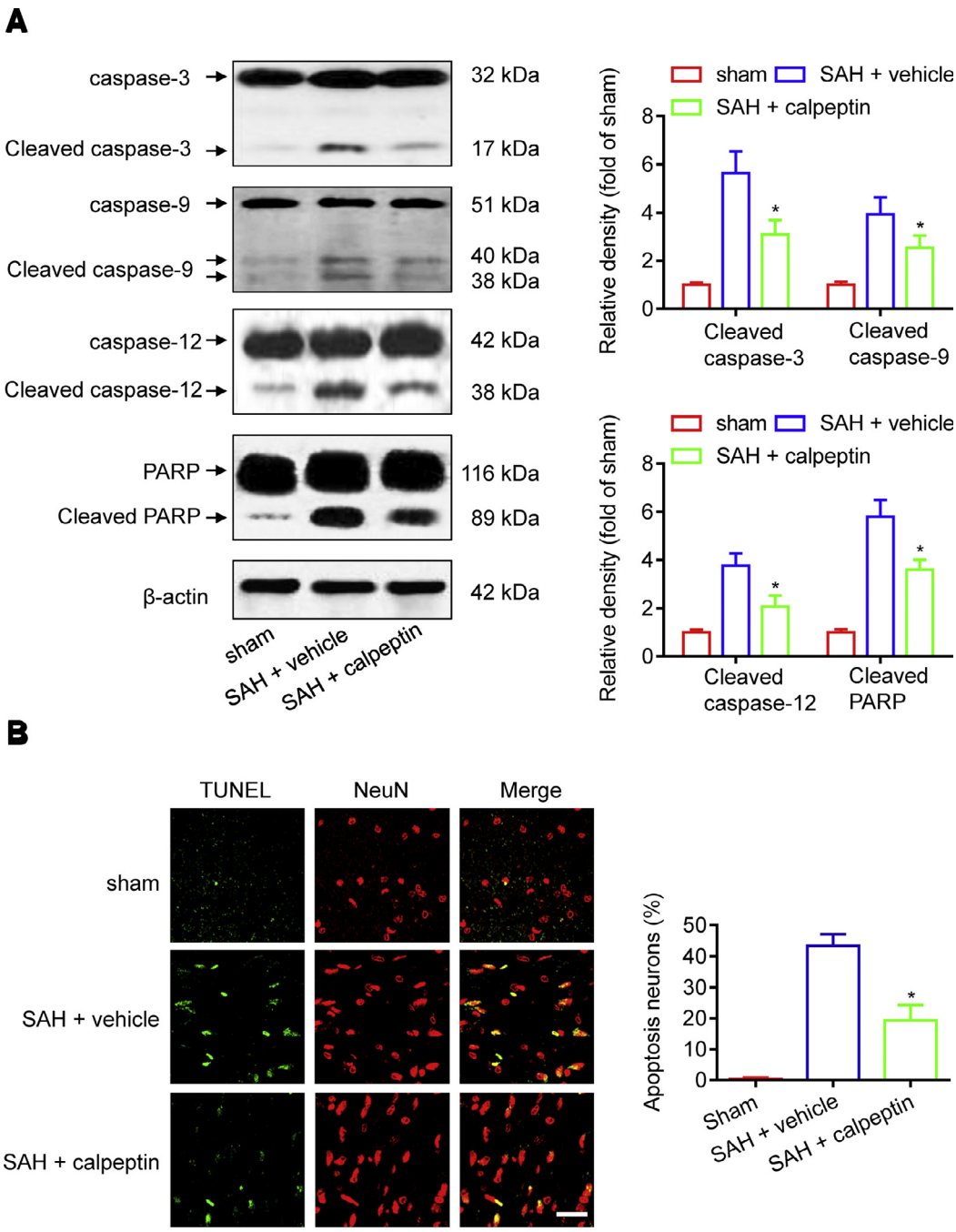

Figure 5. ***Calpeptin attenuated SAH-neuronal apoptosis at day 3.*** *(A) Activation of caspase-3, caspase-9, caspase-12, and PARP. (B) Representative images of TUNEL/NeuN staining of the brain slices in basal cortex were analyzed in sham group, vehicle or calpeptin-treated SAH group (n = 6, P < 0.05 versus SAH + vehicle group).* Scale bar *indicates 50 μm.*

death. In our study, the model of endovascular perforation was used as experimental method for the research of SAH. Using the rat SAH model, the effects of calpeptin on the neurobehavioral deficits and neuronal apoptosis were investigated. Our results indicate that calpeptin improved the neurological deficit after SAH, which is in accordance with previous study in cerebral ischemia,[17] and is also in accordance with previous report describing calpain inhibitor II reduces behavioral deficits.[14] Based on the results obtained from the present study, we suggested that the observed neuroprotective effect is due to calpain inhibition by calpeptin treatment following SAH.

Brain edema is an important feature in early brain injury following SAH and reflects BBB disruption.[22] In the present study, experimental SAH caused brain edema and increase of BBB permeability in rat models. On the other hand, administration of calpeptin significantly attenuated brain edema and BBB permeability in left and

right hemispheres after SAH. We evaluated the tight junction proteins claudin-5, occludin, and ZO-1 expression, which are key components of BBB integrity. Our results showed that calpeptin treatment could restore SAH-induced down-regulation of tight junction proteins. Calpain plays an important role in neuronal death, activated calpain 1 and calpain 2 cleave and activate caspase-12, then activate caspase-9 and caspase-3.[10,23] Our results indicate that calpeptin treatment could reduce cleave of caspase-12, caspase-9, and caspase-3, which consisted with previous study describing calpeptin provides the neuroprotective role through a potential mechanism of caspase-3 inhibition.[17] In this study, we observed calpeptin treatment significantly reduced experimental SAH-elevated the number of TUNEL-positive neurons in basal cortex. These results suggest that inhibition of calpain with calpeptin attenuated neuronal apoptosis in early brain injury after SAH. Even with the limitations of this study, our findings provide information about the effects of inhibition of calpain with calpeptin against SAH-induced neuronal apoptosis in rats.

Conflict of interest

All authors declare no conflict of interest.

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