Icariside II Attenuates Chronic Hydrocephalus in an Experimental Subarachnoid Hemorrhage Rat Model

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ABSTRACT - Purpose To investigate the role of ICA II in subarachnoid hemorrhage (SAH)- related chronic hydrocephalus. **Methods** A two hemorrhage injection model of SAH was created in Sprague Dawley rats (6-8 weeks). A total of 125 rats were randomly assigned into five groups: Sham group, SAH group, SAH+ ICA II (1 mg/kg) group, SAH + ICA II (5 mg/kg) group, and SAH + ICA II (10 mg/kg) group. TGF-β1, phospho-Smad2/3, connective tissue growth factor (CTGF), and procollagen type I carboxy-terminal propeptide (PICP) were assessed via real-time PCR, Western blotting, and enzyme-linked immunosorbent assay. Lateral ventricular index, Masson staining, and Morris water maze tests were employed to evaluate subarachnoid fibrosis, hydrocephalus, and long term neurological function following SAH. **Results** ICA II (1, 5, 10 mg/kg) inhibited subarachnoid fibrosis, attenuated ventriculomegaly, and effectively suppressed SAH related chronic hydrocephalus. In addition, parallel reduced expression of members of the TGF-β1/Smad/CTGF signaling pathway were observed. Importantly, ICA II may improve long term neurocognitive deficits after SAH. **Conclusion** ICA II might suppress fibrosis via inhibition of TGF-β1/Smad/CTGF pathway, prevent the development of SAH related chronic hydrocephalus, and improve long term neurocognitive defects following SAH.

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INTRODUCTION

Among all subtypes of stroke, subarachnoid hemorrhage (SAH) is the most dangerous one, for mortality associated with SAH remains high (40% to 60%), despite some major progress in diagnosis and therapy during the past decades (1). Hydrocephalus is a common medical condition after SAH, characterized by abnormalities in the secretion, circulation, and absorption of cerebrospinal fluid (CSF). Based on clinical and radiographic presentations, post-hemorrhagic hydrocephalus lasting 2 weeks or longer in the course of SAH is defined as chronic hydrocephalus, usually related to subarachnoid space fibrosis, which affects 10% – 20% of SAH patients and requires CSF shunt surgery but still has a high frequency of poor neurological outcomes and cognitive deficits (2-4). Due to the poor understanding of the underlying pathogenesis, there is no efficient treatment and prevention available for chronic hydrocephalus. So, the translation from preclinical research into clinical success is vital and urgent.

Hydrocephalus and brain fibrosis (especially subarachnoid space fibrosis) are common sequelae of whole brain inflammation due to bacterial meningitis, subarachnoid hemorrhage, and severe traumatic brain. It is implied that transforming growth factor-β1 (TGF-β1), a key fibrogenic factor, might play a vital role in the development of chronic hydrocephalus, for the concentration of TGF-β1 increases significantly in the CSF after SAH (5), while only trace amounts of TGF-β1 can be measured in the CSF of healthy humans. It has also been reported that TGF-β1 can contribute to subarachnoid space fibrosis and chronic hydrocephalus via activation of TGF-β1 /Smad/ connective tissue growth factor (CTGF) axis to produce various endogenous cytokines and extracellular matrix (6-8).

Herba Epimedii (Berberidaceae), a traditional Chinese medicine, is used as a tonic remedy for calming the nerve and anti-rheumatic agent in some Chinese proprietary medicine. Icariin ($C_{33}H_{40}O_{15}$, 676.67 Da) and its metabolite IcarisideII(ICA II, $C_{27}H_{32}O_{10}$, 514.54 Da) are the two main bioactive products present in this herb, which have been demonstrated to possess broad therapeutic capabilities, especially anti-osteoporosis, anti-oxidative stress, anti-cancer and neuroprotective effects. In recent years, some preclinical studies have demonstrated neuroprotective effects of ICA II in Alzheimer's

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disease (AD) and ischemic brain injury; it enhanced neurogenesis and promoted memory (9, 10). It can be expected that ICA II may have some protective role in SAH related chronic hydrocephalus for the person who suffers from chronic hydrocephalus will usually have similar cognitive deficits as ischemic brain injury patients.

In this study, ICA II was tested to attenuate chronic hydrocephalus and long-term neurocognitive deficits in an experimental SAH rat model. Although the detailed mechanism still needs to be further explored, given the relatively high incidence and sustained debilitating properties of chronic hydrocephalus, ICA II represents a potentially feasible and promising therapeutic alternative.

METHODS & MATERIALS

Preparation of ICA II
In order to get ICA II (chemical structure shown in Figure 1a), icariin was bio-transformed by the action of β-glucosidase as previous described (11): 50°C, 0.2 M disodium hydrogen phosphate and citric acid buffer system (pH 6.0); the ratio of icariin and enzyme was 1:1; the reaction time was 5 hours; the purification was performed by high-performance liquid chromatography (purity ≥99%). Then, the compound was suspended in 1% carboxymethylcellulose (CMC) to a concentration of 1 mg/ml and fed at the dose of 1, 5, and 10 mg/kg daily respectively.
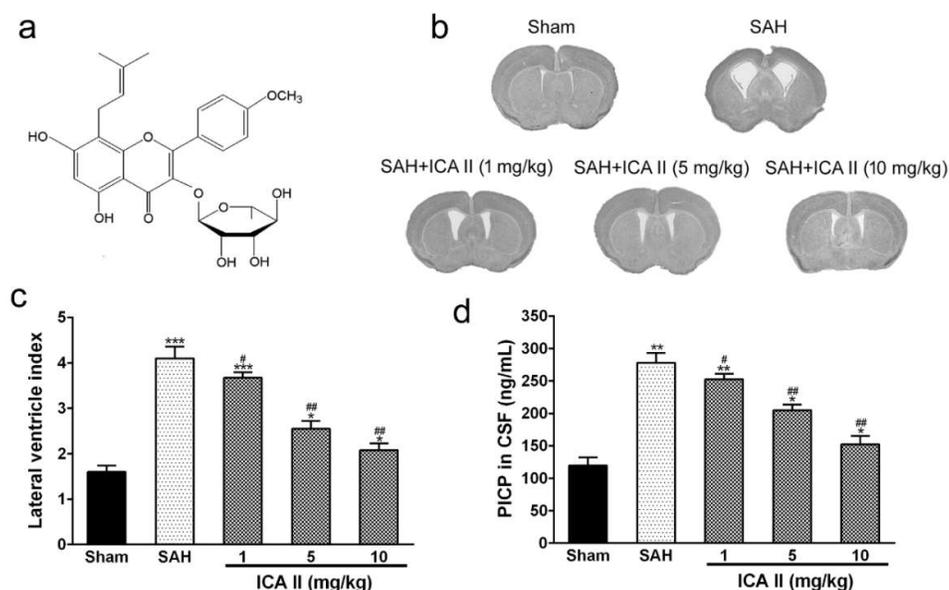
Figure 1. Icariside II reduced chronic hydrocephalus after SAH. (a) Chemical structure of Icariside II. (b) Representative Nissl staining sections of rat brains at the 21st day from different groups. (c) Quantitative analyses of lateral ventricle index at the 21st day after SAH based on Nissl sections of each group. (d) Quantitative analyses of PICP in cerebrospinal fluid at the 21st day after SAH. Data are represented as the mean± SEM of each group. N=15 for each group. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared to sham group; $\#p < 0.05$, $\#\#p < 0.01$ compared to SAH group.

Animals
All experimental protocols were approved by the Ethics Committee of Hebei General Hospital, China. Male Sprague-Dawley rats (6-8 weeks; 160–180 g) of specified pathogen-free grade were purchased from Peking Vital River Laboratory Animal Ltd. (Beijing, China). 125 rats were randomly assigned into five groups (n=25 for each group): Sham group, SAH group, SAH+ ICA II (1 mg/kg) group, SAH + ICA II (5 mg/kg) group, and SAH + ICA II (10 mg/kg) group. All rats underwent oral gavage with saline or ICA II at the dose of 1, 5, or 10 mg/kg daily, respectively (starting 24 hours after SAH surgery).

The administration lasted for 21 days. All rats were fed and maintained in specified pathogen-free conditions and all animal procedures were conducted in accordance with the eighth edition of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011) (12).

SAH model
Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and atropine (0.5 mg/kg, intraperitoneally), and intubated and mechanically ventilated with room air, then placed

in a supine position on a heating pad to keep warm (36.5±0.5°C). The SAH model was set up according to the two-hemorrhage injection method as previously described (13). Briefly, a small (10–15 mm) longitudinal, midline suboccipital incision was cut over the center of the foramen magnum, and the neck muscles were dissected to see the dura. Autologous un-heparinized blood (0.5 ml) drawn from the left femoral artery was injected into the cisterna magna (defined as day 0) using a 25-gauge butterfly needle. The rats were then placed on an inclined board (45° angle) in a neutral position with the head down for 30 minutes. A second blood injection was performed 24 hours later with the same procedure. Sham-operated rats were treated with saline in a similar manner.

Neurobehavioral test
Neurological functions of the SAH rats were evaluated by a combined scoring system including a modified Garcia test and beam balance test (14). The modified Garcia score included spontaneous activity (0–3), spontaneous movement of all limbs (0–3), forepaw out-stretching (0–3), climbing (1–3), body proprioception (1–3), and response to whisker stimulation (1–3). The beam balance test score ranged from 0 to 4 according to the walking distance on a 15 mm-wide wooden beam for 1 minute.

Morris water maze test
Morris water maze test which included cued learning paradigm, spatial paradigm, and probe paradigm (n=15 per group) was performed to evaluate SAH-induced neurocognitive deficits (15). The cued learning trials were conducted on day 18 after SAH and rats were placed in a tank and required to swim to a visible platform above the water surface. On the following 3 consecutive days, the spatial and probe trials were conducted to measure the ability of the rats to learn and remember the location of a hidden platform which was submerged under the water. Once a rat was placed in the tank, it was allowed to swim and search for the platform. The total distance to find the platform was used to reflect spatial learning ability. The platform was removed completely 1 hour after the spatial trial and the rats were allowed to swim again in search of the now-absent platform. The percentage of time spent in the previous location of the platform (the probe quadrant) was utilized to reflect spatial memory ability.

Masson staining and lateral ventricle index calculation
The rats were sacrificed under sodium pentobarbitone anaesthesia. The brain tissues were removed from the dura mater, fixed in 4% paraformaldehyde (4°C, 3 days), and dehydrated with 30% sucrose in phosphate-buffered saline (PBS, pH 7.4). 10 µm serial sections were used to perform Masson staining according to the manufacturer's protocol (Abcam Inc., Cambridge, MA). Briefly, serial sections were stained with Masson staining solution for 5 min, washed with 0.2% acetic acid solution (briefly), 5% phosphotungstic acid (5 min), and 0.2% acetic acid (twice briefly). Then, the serial sections were stained with 1% aniline blue for 5 min, followed by washing with 0.2% acetic acid twice briefly. Finally, the serial sections were dehydrated in absolute alcohol, rendered transparent in xylene, and mounted with mounting medium and analyzed.

The size of the lateral ventricle was determined using the lateral ventricle index, which was calculated as the lateral ventricle volume divided by the total area of the brain section at the level of the preoptic chiasm on the Nissl stained serial sections using ImageJ 1.51p 22 (National Institutes of Health, Bethesda, MD, USA). Hydrocephalus was defined as lateral ventricle index >3 standard deviations above the mean in Sham group (16).

Enzyme-linked immunosorbent assay (ELISA)
On day 21 after SAH, 100 µl CSF was collected and used to detect the concentration of carboxyterminal propeptide of type I procollagen (PICP) and TGF-β1 with relevant ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. All standards and samples were measured with a microplate reader (SpectraMax M5, Molecular Devices) at a wavelength of 450 nm.

Quantitative real-time PCR
Total RNA was extracted from 1 mm thick superficial brain tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was reverse-transcribed from 1 µg RNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). To detect the mRNA levels of TGF-β1 and CTGF, real-time RT-PCR was performed with SYBR Green master mix (Roche, Mannheim, Germany). The reaction procedures were as follows: 95 °C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Expression data were normalized to β-actin mRNA expression. Primer sequences were listed: β-actin, forward primer 5'- ctaaggccaaccgtgaaaag -3', reverse primer 5'- tacatggctggggtgttga -3'; TGF-β1, 5'- aggagcctgctccagagtg-3', reverse primer 5'-agtgacagagtggcaggtca-3'; CTGF, forward primer 5'-tcttctctccaagaagactcagc -3', reverse primer 5'-

gtctggaggaggtcggtct -3'.

Western blotting
The 1 mm thick superficial brain tissues were isolated and lysed and the soluble supernatants (50 µg) were separated by 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. After blocking nonspecific binding with 5% nonfat dry milk, the membranes were incubated with the primary antibodies specific for TGF-β1, CTGF, Smad2/3 and phospho (p)-Smad2/3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 1:1,000 dilution at 4°C overnight. The membranes were then incubated in peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) at a 1:1,000 dilution for 1 h at room temperature and developed with an enhanced ECL system (GE Healthcare Life Sciences, Chalfont, UK). The relative intensity of the proteins of interest was calculated by normalization with β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) from the same sample. Fold changes were then calculated against control intensities from sham-treated animals by densitometry with NIH-Image J1.51p 22 (National Institutes of Health, Bethesda, MD, USA).

STATISTICAL ANALYSIS

Statistical analysis was performed with SPSS (16.0 version) (SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean ± standard error and analyzed by student t test or one-way ANOVA followed by Tukey post-hoc test. Mortality data were analyzed by Fisher exact test. $P<0.05$ was considered to be statistically significant.

RESULTS

Mortality and animal assignment
Of total 125 rats in the present study, 14 rats died within 24 hours after SAH surgery (3 rats in SAH group, the mortality was 12%; 4 rats in SAH + ICA II (1 mg/kg) group, the mortality was 16%; 3 rats in SAH + ICA II (5 mg/kg) group, the mortality was 12%; 4 rats in SAH + ICA II (10 mg/kg) group, the mortality was 16%) . No rat died in the Sham group. In the experiments of Morris water maze, neurobehavioral test and Nissl staining, 15 rats in each group were randomly chosen and we detected the incidence of chronic hydrocephalus after SAH from them. There were 5 rats randomly chosen out from the 15 rats for the experiments of Western blot, ELISA and RT-PCR.

ICA II reduced the incidence of SAH related chronic hydrocephalus
At the 21st day after SAH, ICA II treatment group had a lower incidence of chronic hydrocephalus compared to SAH group (40% versus 46.67% in ICA II 1 mg/kg group; 26.67% versus 46.67% in ICA II 5 mg/kg group; 20% versus 46.67% in ICA II 10 mg/kg group) (Table 1). Nissl staining and quantitative analyses of lateral ventricle index showed that the SAH rats exhibited significantly larger lateral ventricles compared to the ICA II treated rats; while the treatment effects of ICA II were directly proportional to the dose used (Fig. 1b and 1c). PICP, a biomarker for fibrosis, was significantly elevated at the 21st day after SAH in CSF compared to Sham group, and ICA II treatment effectively reduced the level of PICP compared to SAH group (Fig. 1d). All of these findings indicated that ICA II could reduce the incidence of SAH-related chronic hydrocephalus through fibrosis pathway.

Table 1. The incidence of chronic hydrocephalus after SAH.

Experimental group	Hydrocephalus		Incidence
	Yes	No	
Sham	0	15	0
SAH	7	8	46.67%
SAH + ICA II (1 mg/kg)	6	9	40%
SAH + ICA II (5 mg/kg)	4	11	26.67%
SAH + ICA II (10 mg/kg)	3	12	20%

SAH: subarachnoid hemorrhage; ICA II: icariside II

ICA II improved long-term cognitive function in SAH-related chronic hydrocephalus
At the period of day 1 to day 5 after SAH, a significant decrease in neurological scores was detected by comparing SAH group with Sham group. However, the neurological scores of SAH rats gradually increased and showed no differences with Sham rats after day 7 (Fig. 2a). Morris water maze test showed that SAH rats were disabled in latency to reach the platform from the 18th to 20th day after SAH (Fig. 2b) and showed increasing total swim distance when moving to the platform (Fig. 2 c) and decreasing time of entering platform location (Fig.2 d). The ICA II treatment reversed those unfavorable effects compared to SAH group, and the therapy effects were in accordance with the concentration of ICA II (Fig. 2 b–d).

### Increased TGF-β1 and CTGF expression in SAH related chronic hydrocephalus
Not only in CSF but also in brain parenchyma, the

expression of TGF-β1 showed an increased response at the 21st day after SAH when compared with Sham group (Fig. 3 a, b). In addition, the expression of CTGF detected by real-time PCR also demonstrated a significant increase at day 21 after SAH when compared with Sham group in brain parenchyma

(Fig. 3 c). Moreover, ICA II decreased the relative expression of both TGF-β1 and CTGF when compared with SAH group, and such decrease was consistent with the concentration of ICA II (Fig. 3 a, b, and c).
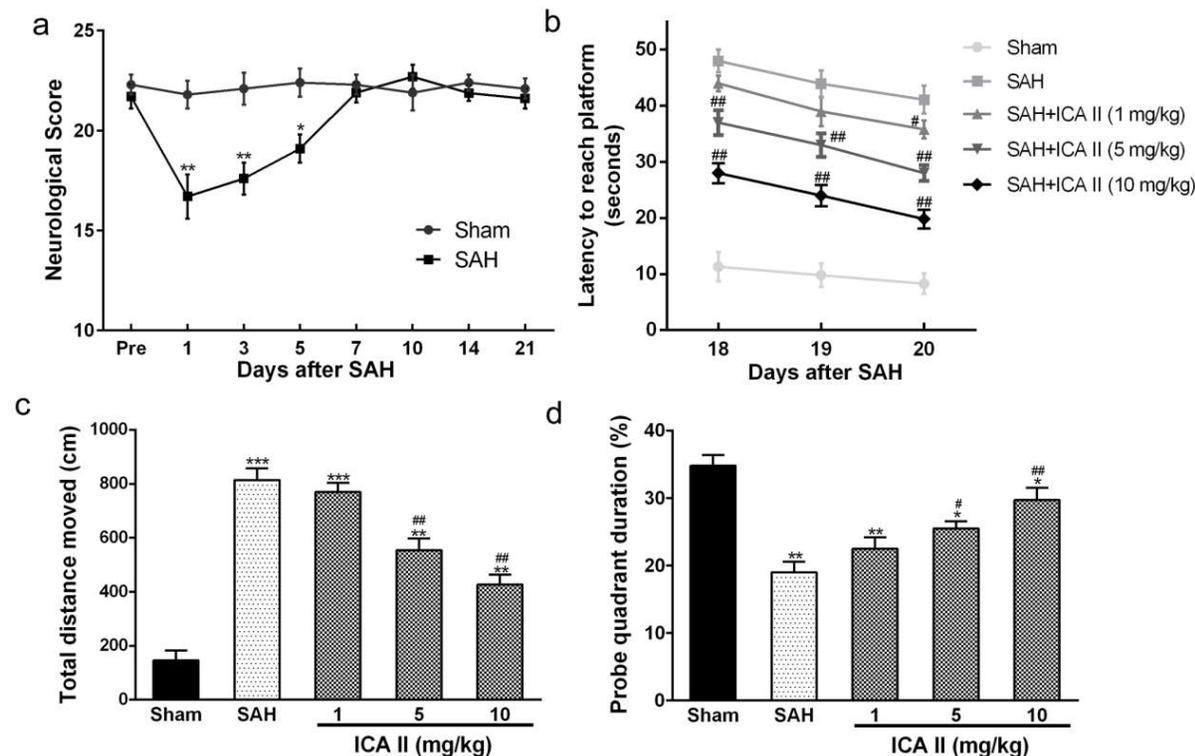
Figure 2. Icariside II improved long-term cognitive functional after SAH. (a) Neurological behavior test of Sham and SAH group in continuous 21 day tests after SAH. (b) Quantitative analyses of the latency to reach the platform from 18th to 20th day after SAH. (c) Quantitative analyses of total swim distance moved to the platform at day 21. (d) Quantitative analyses of relative times of entering platform location at day 21. Data are represented as the mean± SEM of each group. N=15 for each group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to sham group; #$p < 0.05$, ##$p < 0.01$ compared to SAH group.
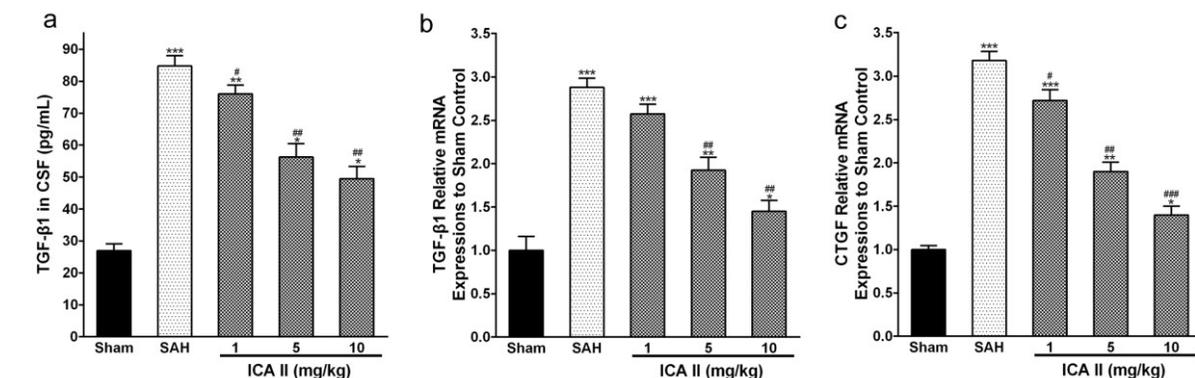
Figure 3. Icariside II suppressed the mRNA expressions of TGF-β1 and CTGF after SAH. (a) Quantitative analyses of TGF-β1 in cerebrospinal fluid at the 21st day after SAH. The mRNA expressions of TGF-β1 and CTGF were assessed by qPCR at the 21st day after SAH (b and c). Data are represented as the mean± SEM of each group. N=5. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to sham group; #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ compared to SAH group.

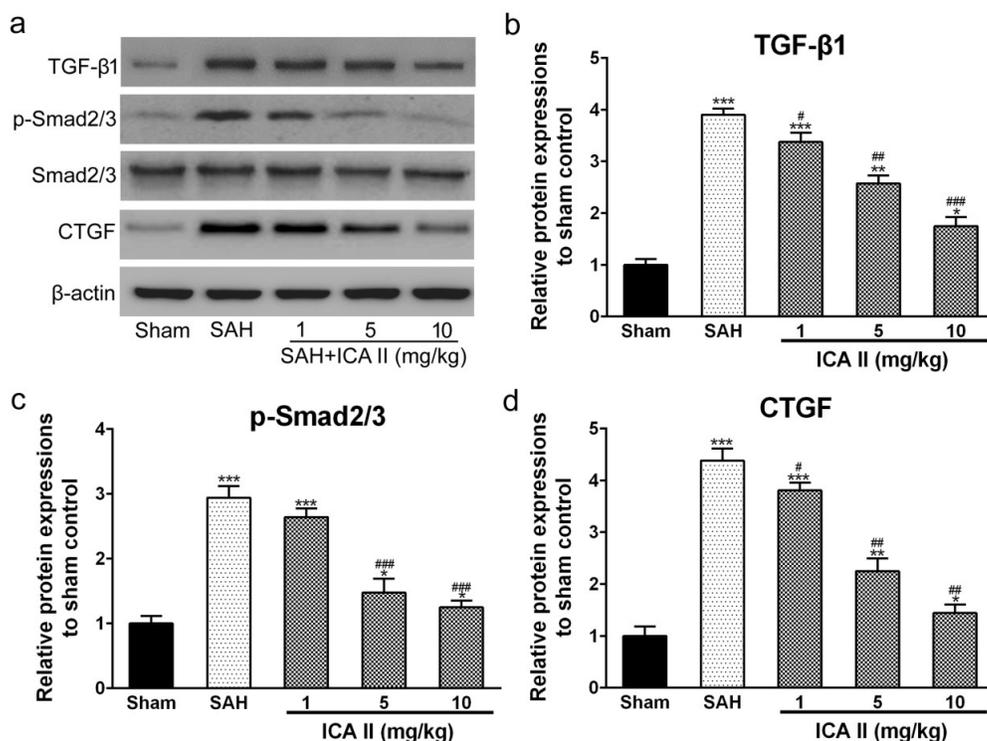**ICA II inhibited the expressions of TGF-β1/Smad/ CTGF after SAH**

To further explore the molecular mechanism involved in the regulation of TGF-β1, the protein levels of TGFβ1, Smad2/3, pSmad2/3, and CTGF were determined in the superficial tissues of rat brain. The expression levels of TGF-β1, Smad2/3, pSmad2/3, and CTGF in SAH group were increased significantly when compared with Sham group. Compared to SAH group, ICA II treatment significantly decreased the upregulation of TGF-β1 and p-Smad2/3 expressions (Fig. 4a, b, and c). Furthermore, ICA II also significantly reduced elevated levels of CTGF after SAH (Fig. 4 a and d). These above results indicated that TGF-β1/Smad/CTGF signaling pathway was involved in the protective effect of ICA II against chronic hydrocephalus.

DISCUSSION

The present study implicates that ICA II can protect against subarachnoid fibrosis, attenuate ventriculomegaly, and effectively suppresses the progress of chronic hydrocephalus in a rat model of SAH. Parallel reduction of TGF-β1 signaling pathway molecules expression indicates that the protective effect of ICA II may be attributed to the inhibition of the activating process of TGF-β1 and downstream Smad2/3 and CTGF signaling pathway, which is critically implicated in the pathogenesis of subarachnoid fibrosis and chronic hydrocephalus after SAH (8). Early brain injury is currently considered the most promising target in the treatment of SAH (17), while delayed neurological deterioration associated with chronic hydrocephalus remains one of the major causes of severe morbidity especially in long-term (18). It is worth noting that the administration of ICA II could ameliorate long-term neurocognitive deficits which happened in SAH related chronic hydrocephalus because the initial latency to reach platform is lower in treated animals, while the learning curve does not seem to be improved (Fig. 2b, same slope between different groups).

Despite the risks, surgery is the most prevalent and efficient therapy to treat chronic hydrocephalus, as potential medicines are still in preclinical status. The medical research community are trying to decipher mechanisms involved in SAH-related chronic hydrocephalus and to investigate more efficient and beneficial treatments for patients.

Figure 4. Icariside II inhibited the expressions of TGF-β1/Smad/CTGF after SAH. (a) Western blotting was used to assay the protein expressions including TGF-β1, p-Smad2/3, Smad2/3 and CTGF. The relative intensity of the bands of interest was calculated by correcting for β-actin (b, c and d). Data are represented as the mean± SEM of each group. N=5. *$p <$ 0.05, **$p < 0.01$ and ***$p < 0.001$ compared to sham group; #$p <$ 0.05, ##$p < 0.01$ and ###$p < 0.001$ compared to SAH group.

One frequently cited theory to explain chronic hydrocephalus after SAH is subarachnoid fibrosis

which is related to CSF outflow obstruction, characterized by abnormalities in the circulation and absorption of CSF which results in ventricular dilatation and contributes to the subsequent development of hydrocephalus (19, 20). The two-hemorrhage injection model of SAH is employed in this study, and ventriculomegaly and fibrosis are observed in our experimental SAH rats, which produce a similar incidence of hydrocephalus as other SAH models. PICP, an important fibrosis factor, is increased significantly at the 21st day after SAH; CTGF, an important amplifier of the pro-fibrogenic action of TGF-β1 in mesenchymal cells and fibroblasts, is elevated significantly in the development of chronic hydrocephalus. All of these data further support the previous assumption that TGF-β1/Smad/CTGF axis contributes to subarachnoid space fibrosis and SAH related chronic hydrocephalus via activation of various endogenous cytokines and stimulation of extracellular matrix synthesis, and SAH related chronic hydrocephalus is a type of fibrotic disease (8, 20, 21). To date, there is no FDA-approved drug for subarachnoid fibrosis. Therefore, in order to improve long-term neurocognitive outcomes of SAH patients, it may be important to develop new therapies against fibrosis.

Herba Epimedii has been recorded in the Chinese medical classics *Shen Nong Ben Cao Jing* 400 years ago and has been broadly used in various traditional Chinese formulations due to its multi-target activity and low toxicity. The herb is proven to have remarkable therapeutic activities to promote osteoblastic proliferation, ameliorate streptozotocin-induced diabetic nephropathy in diabetic rats, alleviate ischemia-reperfusion-induced hippocampal injury in gerbils, and inhibit the growth of the various cancer cell lines (22). ICA II, an active flavonoid glycoside derived from *Herba Epimedii*, also has multiple effects on various diseases, especially some notable protective effects on cognitive deficits and cerebral ischemia-reperfusion injury (9). Although the precise mechanism involved still needs further study, ICA II might be a potential candidate to treat SAH related chronic hydrocephalus by inhibiting the TGF-β1/Smad/CTGF pathway.

It is worth mentioning that there may be other mechanisms involved in SAH related chronic hydrocephalus, such as angiographic vasospasm, arteriolar constriction, thrombosis, and cortical spreading ischemia (18).. What makes it more complex, many patients survive acute attacks of SAH but deteriorate days later from chronic hydrocephalus, which causes poor outcome or death in up to 30% of persons with SAH. Emerging data on secondary brain ischemia after SAH and some common signaling pathways and therapeutic targets relating ischemia/reperfusion injury and hydrocephalus may offer a novel therapeutic target. It is reported that ICA II protects against cerebral ischemia/reperfusion injury and alleviates the microcirculatory disturbance and neuronal injury via up-regulation of PPARα and PPARγ and inhibition of NF-κB activation (9). In addition to that, ICA II has been found to induce apoptosis in various human cancer cell lines of different origin by targeting multiple signaling pathways including STAT3, PI3K/AKT, MAPK/ERK, COX-2/PGE2 and β-Catenin which are frequently deregulated in cancers (23). Whether such mechanisms are also involved in the ICA II treatment of SAH related chronic hydrocephalus needs to be further explored.

Our goals in the present study were to determine the treatment potential of ICA II in SAH related chronic hydrocephalus, to further understand the pathophysiological mechanisms involved in hydrocephalus, and to promote the development of better therapies. Mostly because the pathogenesis of SAH related chronic hydrocephalus is frequently multifactorial (24), the multi-target characteristics of traditional Chinese medicine may be advantageous over single-target drug in the treatment of complex diseases. Although the results of the present study need to be confirmed in future basic research and larger clinical trials, treatment with ICA II holds promising potential.

CONCLUSION

ICA II shows promise in the treatment of SAH-related chronic hydrocephalus, which may modulate subarachnoid fibrosis through TGF-β1/Smad/CTGF signaling pathway.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

FUNDING

REFERENCES

1. Yamada S, Koizumi A, Iso H*, et al.* Risk factors for fatal subarachnoid hemorrhage: the Japan Collaborative Cohort Study. Stroke, 2003; 34: 2781.
2. Dupont S and Rabinstein AA. Extent of acute hydrocephalus after subarachnoid hemorrhage as a risk factor for poor functional outcome. Neurological Research, 2013; 35: 107-110.
3. O'Kelly CJ, Kulkarni AV, Austin PC*, et al.* Shunt-dependent hydrocephalus after aneurysmal subarachnoid hemorrhage: incidence, predictors, and revision rates. Clinical article. Journal of Neurosurgery, 2009; 111: 1029.
4. Woernle CM, Winkler KM, Burkhardt JK*, et al.*

Hydrocephalus in 389 patients with aneurysm-associated subarachnoid hemorrhage. Journal of Clinical Neuroscience, 2013; 20: 824-826.

5. Liu F, Yuan W, Liao D, *et al.* [Association of chronic hydrocephalus after aneurysmal subarachnoid hemorrhage with transforming growth factor-β1 levels and other risk factors]. Journal of Southern Medical University, 2013; 33: 382-385.

6. Ito Y, Goldschmeding R, Kasuga H, *et al.* Expression patterns of connective tissue growth factor and of TGF-beta isoforms during glomerular injury recapitulate glomerulogenesis. Am. J. Physiol. Renal Physiology, 2010; 299: F545-558.

7. Nagaraja T, Chen L, Balasubramanian A, *et al.* Activation of the Connective Tissue Growth Factor (CTGF)-Transforming Growth Factor β 1 (TGF-β 1) Axis in Hepatitis C Virus-Expressing Hepatocytes. Plos One, 2012; 7: e46526.

8. Yan H, Chen Y, Li L, *et al.* Decorin alleviated chronic hydrocephalus via inhibiting TGF-β1/Smad/CTGF pathway after subarachnoid hemorrhage in rats. Brain Research, 2016; 1630: 241.

9. Deng Y, Xiong D, Yin C, *et al.* Icariside II protects against cerebral ischemia-reperfusion injury in rats via nuclear factor-κB inhibition and peroxisome proliferator-activated receptor up-regulation. Neurochemistry International, 2016; 96: 56-61.

10. Xiong D, Deng Y, Huang B, *et al.* Icariin attenuates cerebral ischemia-reperfusion injury through inhibition of inflammatory response mediated by NF-κB, PPARα and PPARγ in rats. International Immunopharmacology, 2016; 30: 157-162.

11. Xia Q, Xu D, Huang Z, *et al.* Preparation of icariside II from icariin by enzymatic hydrolysis method. Fitoterapia, 2010; 81: 437-442.

12. Council N. Guide for the Care and Use of Laboratory Animals: Eighth Edition. Guide for the Care & Use of Laboratory Animals, 2011; 327: 963-965.

13. Jian Z, Xiang X, Dai Z, *et al.* Possible Role of Raf-1 Kinase in the Development of Cerebral Vasospasm and Early Brain Injury After Experimental Subarachnoid Hemorrhage in Rats. Molecular Neurobiology, 2015; 52: 1527.

14. Chen Y, Zhang Y, Tang J, *et al.* Norrin protected blood-brain barrier via frizzled-4/β-catenin pathway

.

after subarachnoid hemorrhage in rats. Stroke, 2015; 46: 529-536.

15. Hu Q, Liang X, Chen D, *et al.* Delayed hyperbaric oxygen therapy promotes neurogenesis through reactive oxygen species/hypoxia-inducible factor-1α/β-catenin pathway in middle cerebral artery occlusion rats. Stroke, 2014; 45: 1807.

16. Okubo S, Strahle J, Keep RF, *et al.* Subarachnoid Hemorrhage-Induced Hydrocephalus in Rats. Stroke, 2013; 44: 547-550.

17. Sehba FA, Hou J, Pluta RM, *et al.* The importance of early brain injury after subarachnoid hemorrhage. Progress in Neurobiology, 2012; 97: 14.

18. Macdonald RL. Delayed neurological deterioration after subarachnoid haemorrhage. Nature Reviews Neurology, 2014; 10: 44.

19. Suzuki H, Kinoshita N, Imanakayoshida K, *et al.* Cerebrospinal fluid tenascin-C increases preceding the development of chronic shunt-dependent hydrocephalus after subarachnoid hemorrhage. Stroke, 2008; 39: 1610-1612.

20. Hao X, Junwen W, Jiaqing L, *et al.* High fibrosis indices in cerebrospinal fluid of patients with shunt-dependent post-traumatic chronic hydrocephalus. Translational Neuroscience, 2016; 7: 92-97.

21. Flood C, Akinwunmi J, Lagord C, *et al.* Transforming growth factor-beta1 in the cerebrospinal fluid of patients with subarachnoid hemorrhage: titers derived from exogenous and endogenous sources. Journal of Cerebral Blood Flow & Metabolism, 2001; 21: 157-162.

22. Yin C, Deng Y, Gao J, *et al.* Icariside II, a novel phosphodiesterase-5 inhibitor, attenuates streptozotocin-induced cognitive deficits in rats. Neuroscience, 2016; 328: 69.

23. Muhammad K, Amara M, Javed Iqbal Q, *et al.* Targeting Apoptosis and Multiple Signaling Pathways with Icariside II in Cancer Cells. International Journal of Biological Sciences, 2015; 11: 1100-1112.

24. Chao Z, Lei Y, Geng YD, *et al.* Icariside II, a natural mTOR inhibitor, disrupts aberrant energy homeostasis via suppressing mTORC1-4E-BP1 axis in sarcoma cells. Oncotarget, 2016; 7: 27819-27837.