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ORIGINAL ARTICLE

Neuroprotective roles of total flavones of Camellia on early brain injury andcognitive dysfunction following subarachnoid hemorrhage in rats

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Abstract

The present study was undertaken to explore the role of total flavones of Camellia (TFC) on cerebral injury following subarachnoid hemorrhage (SAH) in rats. We showed that the increase of malondialdehyde (MDA) level in brain tissues, leakages of neuron-specifc enolase (NSE) and lactate dehydrogenase (LDH) from brain tissues to serum at 48 h after SAH were significantly blocked by TFC treatment. Besides, TFC treatment could reduce brain edema and the Bax/Bcl-2 ratio in hippocampal tissues at mRNA and protein levels at 48 h after SAH. In addition, and the reduction of neurological scores at 7d after SAH were significantly inhibited by TFC treatment. We next sought to demonstrate the role of TFC on cognitive rehabilitation and the tau phosphorylation in hippocampal tissues at 30d after SAH. Not surprisingly, cognitive dysfunction and the upregulation of tau phosphorylation at Ser262 (p-tau-Ser262) in hippocampal tissues were markedly reduced by TFC treatment. These findings suggested that TFC has protective effect on SAH-induced EBI and subsequent cognitive dysfunction, which may be related to downregulating the Bax/Bcl-2-related apoptosis pathway and inhibition of tau phosphorylation.

Keywords TFC · SAH · Learning and memory function · Bax/Bcl-2 · Tau

Abbreviations

TFC	total flavones of Camellia
SAH	Subarachnoid hemorrha
EBI	early brain injur
MDA	malondialdehyde
NSE	neuron-specifc enolase
LDH	lactate dehydrogenase
CJ	Camellia japonica L.
ECJ	Camellia japonica L. flower
MWM	Morris water maze
RT-PCR	Reverse Transcription Polymerase chain reaction

Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is one of common causes of neurological disorder with high morbidity and mortality. Despite the progress in surgical techniques applied in clinical, the outcome of patients with SAH remains unsatisfactory (Elmaraezy et al. 2017; Wang et al. 2013), the early brain injury (EBI) in particular is the main cause of patients' unsatisfactory outcome (Yuksel et al. 2012). Multiple factors are involved in the EBI induced by SAH, such as oxidative stress, abnormal regulation of inflammatory responses and cerebral vasospasm (Yu et al. 2014). Approximately half of SAH patients die of EBI, many of survivors are left with lasting cognitive dysfunction and deficits, which are further accompanied with anxiety, sleep disturbances and depression. Therefore, the inhibition of cognitive deficits has been considered as one of vital targets in the therapy of SAH survivors(Al-Khindi et al. 2010).

As previously reported, many plant extracts and/or constituents have been considered as possible therapeutic approach to treat neurodegenerative and cognitive disorders (Jurado-Coronel et al. 2016; Kumar and Khanum 2012). Camellia japonica L. (CJ) is known as a member of the tea family and

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widely used as cosmetic sources, and possesses a diversity of bioactivities, such as antioxidant activity(Mizutani and Masaki 2014; Onodera et al. 2006; Woo et al. 2017)and endothelium-dependent vasodilation (Kumar and Khanum 2012; Park et al. 2015). We previously revealed the protective role of extract of Camellia japonica L. flower (ECJ) on cerebral ischemia-reperfusion injury in rats (Lu et al. 2019), and the protection of total flavones of Camellia (TFC) on cerebrovascular dysfunction injured by SAH (Lu and Wen 2019). Therefore, we hypothesized that the cerebrovascular protection of TFC may be involved in neuroprotection following SAH.

In view of the fact that NSE is more specific for neuronal injury, and serum NSE activity is a sensitive biomarker for the degree of brain injury (Toklu et al. 2018). In addition, oxygen-free radicals in neurocytes induced by cerebral injury could subsequently cause lipid peroxidation and cerebral damage. Hence, like NSE and LDH leakage from injured cells to serum, MDA, a product of lipid peroxidation, has also been applied to assess cerebral injury (Liang et al. 2015).Thus, in present study, serum NSE and LDH activity and MDA level in brain tissues were used to assess the role of TFC on the EBI after experimental SAH. Likewise, we followed the same experimental SAH approach to further explore the mechanism of protective effect of TFC via detecting the Bax/Bcl-2 ratio at RNA and protein levels, and phosphorylated tau in hippocampal tissues.

Materials and methods

Animals

Adult Sprague Dawley rats (male to female: 1:1, 220–250 g) were purchased from Animal Center of Anhui Medical University. Animals were housed and habituated to the novel environment for one week under standard laboratory conditions, with a controlled temperature 22 ± 2 °C and relative humidity 54 ± 2%, with free to food and water. All experimental procedures conformed to the Ethics Review Committee of Hefei technical college, which follow the protocol outlined in the Guide for the Care and Use of laboratory Animals published by the US National Institutes of Health (NIH publication number 86 − 23, revised 2011).

Drugs and reagents

The TFC(content of flavones over 85%) was provided by the department of Pharmacology of Anhui Medical University (Mingyu Huang 2012). MDA, NSE and LDH test kits were purchased from Nanjing Jiancheng Bilolgical Company (Nanjing, China).

Experimental paradigms

The Sprague Dawley rats were randomly divided into six groups: sham group, SAH group, SAH + saline group, SAH + TFC (80 mg/kg) group, SAH + TFC (40 mg/kg) group, SAH + TFC (20 mg/kg) group. TFC or equal volume of saline was administrated using an intragastric method once a day from experimental SAH day. The dosage of TFC was decided according to our previous studies (Weizhuo Lu et al. 2017). In the first part of experiments, the SAH rats were sacrificed by decapitation under deep anesthesia of isoflurane at 48 h after SAH, the sera and brain tissues were collected. And then, MDA level and Bax/Bcl-2 ratio in hippocampal tissues, the leakage of NSE and LDH from injured brain tissues to serum, and brain edema were detected. In the second experimental setting, the rats were used to assess the neurological scores at 7d after SAH. Besides, the Step down test and Morris water maze (MWM) were used to evaluate the cognitive dysfunction at 30 days after SAH, after that the rats were sacrificed under deep anesthesia for detection of p-tau-Ser262 in hippocampal tissues.

SAH model

As described previously (Lu and Wen 2019), rats were deeply anesthetized with isoflurane, and fixed on a heated operation table to maintain body temperature at around 37°C. A catheter was introduced into the femoral artery to withdraw blood and to measure blood pressure. Nearly 1.0cm midline scalp incision was prepared and a hole with diameter of 2.0 mm was drilled with 7.5 mm anterior to the bregma through the skull in the midline after the disinfection. Autologous blood (200μL) was withdrawn from the femoral artery and then injected into the cisterna magna through the prepared hole with needle over a three minutes period. The needle kept in this position for at least 2 min to prevent cerebrospinal fluid (CSF) or leakage blood backflow after injection. The drilling point sealed with bone wax and the sterile incision sutured. Sham-operated rats underwent the same procedures, but injection with equal volume of normal saline solution.

Evaluation of neurological scores

At 7d after SAH, neurological scores were evaluated based on a modified Garcia scoring system (Hasegawa et al. 2011). (1) scores 0–3: spontaneous activity; (2) scores 1–3:reaction to side stroking; (3) scores 1–3:reaction to vibrissae touch; (4) scores 0–3:limb symmetry;(5) scores 0–3:forelimb outstretching; (6)scores 0–3: climbing (7) scores 0–4: beam walking abilities. The total scores of above seven subtests were used to evaluate neurological function. Lower scores indicated worse neurological function, and higher scores reflected better function.

Determination of brain water content

The rats were sacrificed at 48 h after SAH by decapitation under deep anesthesia and the brains were rapidly removed. The dry-wet approach was used to measure the brain water content (Wen et al. 2019). In short, fresh brain was weighed to attain the wet weight. Then, the fresh tissue was dried in an oven at 105 °C for 48 h and weighed again to obtain the dry weight. Brain water content was calculated with following formula:

Measurement of serum LDH and NSE activity, brain tissue MDA level

Rat sera and brains were harvested at 48 h after SAH. The brains were washed twice with cold saline solution and then homogenized. The supernatant of homogenate of brain tissues were collected after centrifugation. and the sera and supernatant of homogenate of brain tissues were respectively transferred to 96 well plates for LDH and NSE activity and MDA level analysis using the biochemistry assay kit (Jiancheng Bioengineering Ltd, Nanjing, China) and abiding by the manufacturer's manual.

Reverse Transcription Polymerase chain reaction (RT-PCR)

Total RNA was extracted from the hippocampal tissues with TriPure Isolation Reagent (Roche, Basel, Switzerland). And then, the one-step reverse transcription PCR was conducted (Wen et al. 2016). The primers of Bcl-2 were 5'-TGAACCGG CATCTGCACAC-3' and5'-CGTCTTCAGAGACAGC CAGGAG-3'; the primers of Bax were 5'-AGACACCT GAGCTGACCTTGGAG-3' and 5'-GTTGAAGTTGCCAT CAGCAAACA-3'. β-actin mRNA was used as internal control and amplified with a pair of primers:5'-GTCCCTCA CCCTCCCAAAAG-3'. and 5'-GCTGCCTCAACACC TCAACCC-3'. All PCR products were electrophoresed on a 1% (w/v) agarose gel, stained with ethidium bromide and photographed under UV light. The Image-ProPlus 6.0 software was used to determine the intensity of bands. The densitometric quantification of these values was normalized to β-actin.

Western blot assay

The total proteins in hippocampal tissues were extracted and separated by 12% SDS-PAGE as previously described (Wen et al. 2019), and then electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked for 1 h with tris-buffer saline containing 5% skim milkand subsequently incubated with Bax,Bcl-2, p-tau- Ser 262 antibody (Santa Cruz Biotechnology, ca., USA) or β-actin antibody (1:2000; Sigma-Aldrich)in same buffer overnight at 4 °C, respectively. The β-actin in the same protein extractswas used as an internal control. After washing for six times, the horseradish peroxidase-conjugated anti-rat IgG (KPL, Gaithersburg, MD, USA) was added at a dilution of 1:2000 in PBS containing 5% skim milk. The densitometry of the band was used to determine the relative intensity.

Step down test

At 30d after SAH, experimental rat was placed in the jumping apparatus with a platform (Heo et al. 2014; Wen et al. 2019; Zhu et al. 2014). After adapting for 5 min, rat received foot shock of 36V electric from the floor. The latency of rat stepping onto the elevated platform was recorded as learning latency, the frequency of it stepping down from elevated platform within 5 min was recorded as number of learning errors. At 24 h later, step down test was carried out again, the latency and frequency of rat receiving electric shocks within 5 min was assessed as the memory latency, and number of memory errors, respectively.

Morris water maze(MWM) test

The MWM test was undertaken after Step down test. MWM includes a diameter 150 cm and height 60 cm circular pool and a platform. The pool filled with water (21–24 °C) was divided into NE, SE, SW and NW equal quadrants. The platform was submerged 1 cm below the water surface and set in the SW quadrant. The water was made into milk-white by adding nontoxic titanium dioxide powder, where rats could not see the platform directly. Movement of rats in the maze was monitored by a computerized video tracking system (Liu et al. 2007; Su et al. 2009; Zhu et al. 2014).The MWM test consisted two phases: place navigation and spatial probe. In place navigation test, rat was set into the water at starting points of the one of four quadrants, and allowed to swim freely to find the hidden platform. The time taken to escape on the platform was recorded as escape latency. If rat failed to locate the platform within 60 s, it was guided on the platform and allowed to stay there for 20 s, and its escape latency was recorded as 60 s. Subsequent starting points for the next three trials carried out in a clockwise direction.

Twenty-four hours after place navigation test, the platform was removed from the pool, and then the probe test was performed to measure the numbers of entry, time and distance of rat crossing the quadrant where the platform was withdrawn.

Data analysis

The data are expressed as mean ± SD. Statistical analyses were performed with one-way ANOVA followed by the Tukey's method to determine the difference between groups. A value of $p < 0.05$ was considered statistically significant.

Results

Effect of TFC on brain water content and neurological behaviors after SAH

The brain water content increased markedly at 48 h after SAH in SAH group (compared with sham group, $p < 0.01$), which could be significantly attenuated by 80,40 mg/kg TFC treatment (compared with SAH group, $p < 0.01$) (Fig. 1a). Moreover, the neurological dysfunction in SAH group at 7d after SAH was more significant (compared with sham group, $p < 0.01$). Whereas,TFC treatment (80,40 mg/kg) significantly ameliorated the neurological deficits injured by experimental SAH (compared with SAH group, $p < 0.01$)(Fig. 1b).

Effect of TFC on SAH-induced changes of serum NSE and LDH, cerebral tissue MDA

Increases of NSE and LDH activity in serum for those leakages from brain cells (Kanavaki et al. 2017; Wang et al. 2019) and MDA, a product of lipid peroxidation, have been applied to assess cerebral I/R injury. As shown in Fig. 2, the remarkable increases of serum NSE and LDH activity were detected (compared with sham group, $p < 0.01$, respectively). Besides, the MDA content in cerebral tissues was also more significant in the SAH group. TFC (40, 80 mg/kg) treatment could remarkedly reduce theMDA Level in brain tissues and NSE and LDH activity in sera.

Effect of TFC on SAH-induced changes of Bax/Bcl-2 ratio

The changes of Bax and Bcl-2 in hippocampal tissues were examined at mRNA and protein levels at 48 h after SAH. The results showed in Fig. 3, the Bax/Bcl-2 ratios both at mRNA and protein levels increased markedly after SAH. Whereas, the increment of Bax/Bcl-2 ratios were significantly reduced by 80 mg/kg TFC treatment (compared with SAH group, $p < 0.01$).

Effect of TFC rat learning and memory deficits

As shown in Fig. 4, the results of step down test showed that the learning latency and mistake numbers of learning and memory significantly increased in SAH group rat at 30d after SAH, on the contrary, the memory latency obviously decreased in SAH group. These results indicated that experimental SAH could induce learning and memory deficits in rats, which could be significantly alleviated by 80 mg/kg TFC treatment.

Furthermore, the results of effect of TFC on the rat place navigation and spatial probe ability after SAH showed that

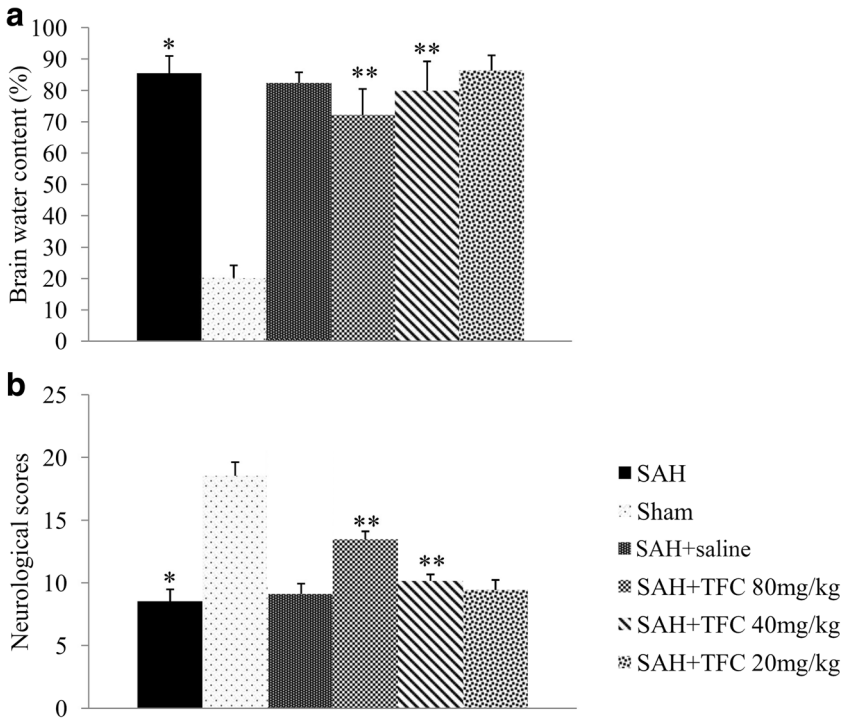

Fig. 1 TFC ameliorated SAH-induced brain injury(means ± SD, n = 08). **a** Effect of TFC on changes of brain water content measured at 48 h after SAH, **b** Effect of TFC on changes of neurological scores at 48 h after SAH, *$p < 0.01$ vs. Sham, **$p < 0.01$ vs. SAH

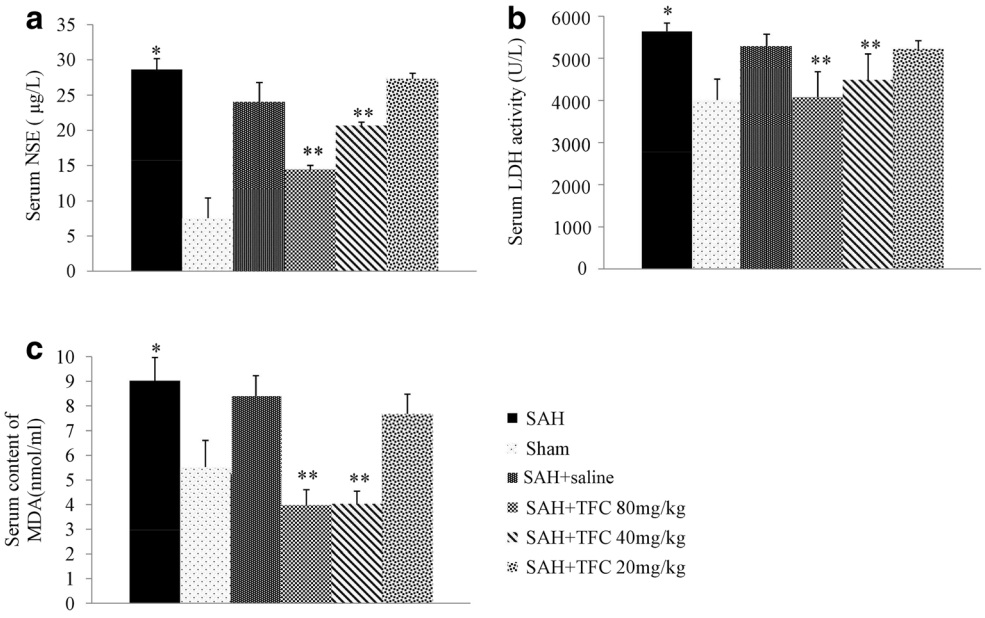

Fig. 2 Effect of TFC on SAH-induced changes of NSE and LDH in rat serum and MDA in rat brain tissues (means ± SD, n = 08). **a** Effects of TFC on the changes of the serum NSE in rats, **b** Effects of TFC on the changes of serum LDH in rats, **c** Effects of TFC on the changes of MDA content in rat brain tissues, *$p < 0.01$ vs. Sham, **$p < 0.01$ vs. SAH

there was no obvious difference of the escape latencies among all groups on day 1 training. The performance of all rats got improvement with training from day 2 to 4. But the escape latencies of the SAH group rats were markedly longer than those of the sham group on day 3 and 4, suggesting a SAH-induced damage of place navigation ability (Fig. 5). Figure 5 also showed that 40,80 mg/kg TFC could obviously ameliorate the damage of rats navigation bility injured by SAH.

Moreover, the impairment of spatial probe ability injured by SAH was assessed by decreases of number of entry, time and distance of crossing platform location. The results revealed that effect of SAH on rats spatial probe ability was similar to that on the place navigation ability, both proportions of time and distance and number of entry in the SAH group rat decreased evidently compared to those in the sham group. Similarly, 40, 80 mg/kg TFC had obvious therapeutic effection on SAH rats (Table 1).

Effect of TFC on Tau-Ser262 phosphorylation in rat hippocampal tissues

Western blot was used to assess the phosphorylation of tau at Ser262(p-tau-Ser262 within the microtubule binding region.

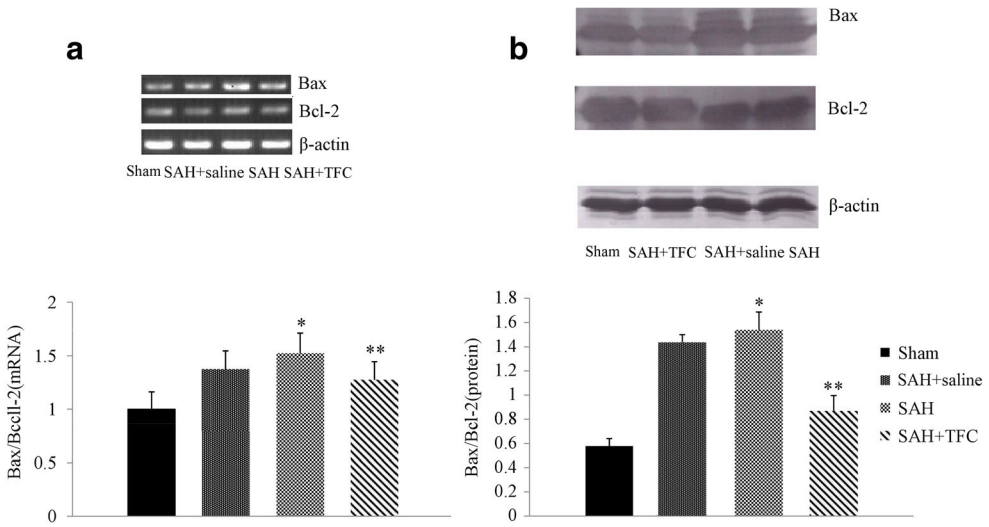

Fig. 3 Effects of TFC on the expressions of Bax and Bcl-2 at the mRNAand protein levels (n = 04). **a** The relative expression levels of Bax and Bcl-2 mRNA in the hippocampal tissues. The mRNA were quantified by calculating the densitometric ratio of the bands of Bax and Bcl-2 to β-actin (mean ± SD, n = 04), **b** Expression of Bax and Bcl-2 (Western blot method, mean ± SD, n = 04),Bax and Bcl-2 were quantified by calculating the densitometric ratio of the bands of Bax and Bcl-2 to β-actin. The mRNA and protein levels were obtained from four independent experiments. *p < 0.01,vs. Sham, **p < 0.01 SAH

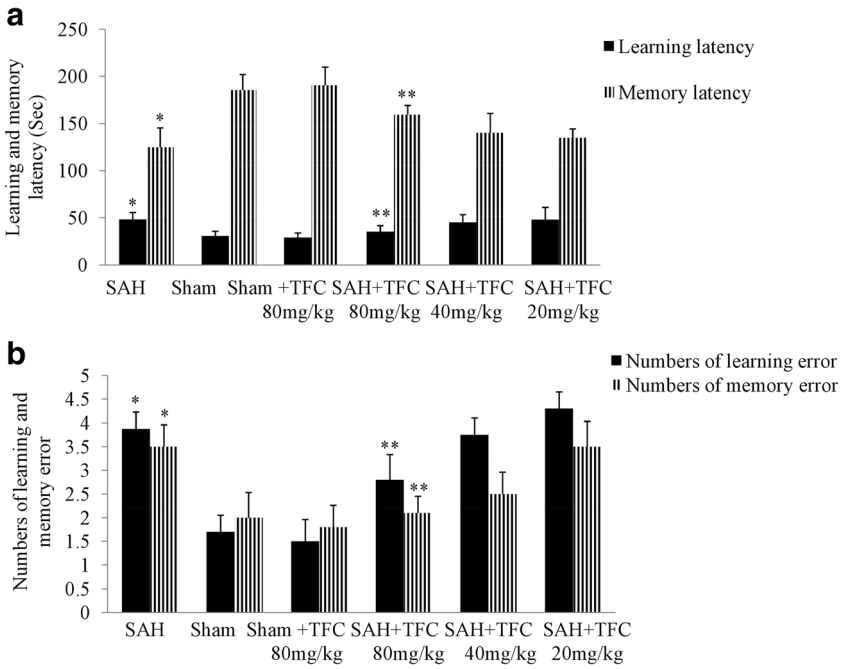

Fig. 4 Effect of TFC on SAH-caused learning and memory deficits in rats (Step down test, means ± SD, n = 08). **a** Learning and memory latencies (Sec), **b** Numbers of learning and memmory errors, *p < 0.01,vs. Sham, **p < 0.01 SAH

We found that the p-tau-Ser262 was significantly elevated in hippocampal tissues of SAH rats. In contrast, the p-tau-Ser262 was reduced by TFC treatment (Fig. 6, compared with SAH group, p < 0.01).These results indicated that inhibition of tau phosphorylation may be responsible for the protection of TFC on cognitive dysfunction induced by SAH.

Discussion

Accumulated evidences have suggested that EBI, which occurs at the 24–72 h after SAH, mainly contributes to unfavorable outcome of patients, such as a mortality rate of more than 50%, lasting cognitive dysfunction and impaired day to day functioning (Bederson et al. 2009). The treatment of EBI is a primary aim in the management of SAH survivors. Thus, the main priority is to demonstrate the mechanisms of EBI, explore the neuroprotective strategies and find new drug for possible clinical use.

Herbal medicine is regarded as the bench mark for multi-target therapy with promising future (Gao et al. 2016). Traditional Chinese medicines have been found to be used to treat various diseases for over thousands of years (Chang et al. 2007). A few of plant-derived natural active ingredients have been reported to be involved in protection on brain injury after SAH (Lan et al. 2017; Shi et al. 2017). We previously

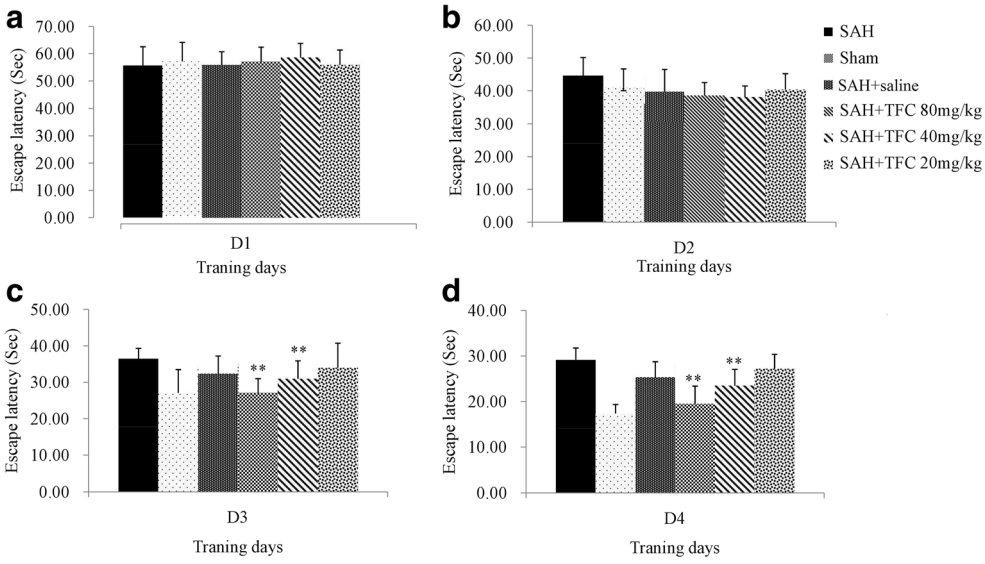

Fig. 5 Effect of TFC on SAH-caused impairment of mouse place navigation ability (Morris water maze test, means ± SD, n = 08). **a** Training day (1), **b** Training day (2), **c** Training day (3), **d** Training day 4, *p < 0.01,vs. Sham, **p < 0.01 SAH

Table 1 Effect of TFC on impairment of rat spatial probe ability induced by SAH

Group	Dose(mg/kg)	Number of entries	Proportion of time (%)	Proportion of swim distance (%)
SAH	/	1.25 ± 0.45*	20.15 ± 2.11*	26.31 ± 1.32*
Sham	/	2.45 ± 0.54	40.94 ± 3.15	35.12 ± 1.21
Sham + TFC	80	2.76 ± 0.22	43.1 ± 0.39	35.2 ± 1.35
SAH + TFC	80	2.25 ± 0.25**	31.47 ± 1.07**	32.15 ± 1.45**
SAH + TFC	40	1.87 ± 0.11**	28.4 ± 0.08**	31.5 ± 1.98**
	20	1.33 ± 0.29	21.22 ± 1.11	28.73 ± 1.11

* $P < 0.01$ vs. Sham; ** $P < 0.01$ vs. SAH

also found the protective effect of TFC on the cerebrovascular dysfunction (Lu and Wen 2019). Thus, the main priority of present study was undertaken to explore the role of TFC on SAH-induced cerebral injury.

Previous studies have reported that the mechanisms of EBI after SAH may include the reduction in cerebral perfusion pressure, the rapid rise of intracranial pressure, brain edema, oxidative stress, and blood-brain barrier (BBB) disruption (Qu et al. 2017; Sehba et al. 2012). Rise of intracranial pressure produces large amounts of oxygen-free radicals in neurocytes,causes lipid peroxidation and cerebral damage. Hence, MDA, LDH and NSE leakage from neuron have been applied to assess cerebral injury (Kanavaki et al. 2017; Wang et al. 2019). In the present study, we found that LDH and NSE activity in serum and MDA content in cerebrum of rat significantly increased at 48 h after experimental SAH, suggesting a cerebral injury in early phase of SAH, which could be significantly ameliorated by TFC (40 and 80 mg/kg) treatment. These data indicated that TFC treatment could inhibit the SAH-induced cerebral injury.

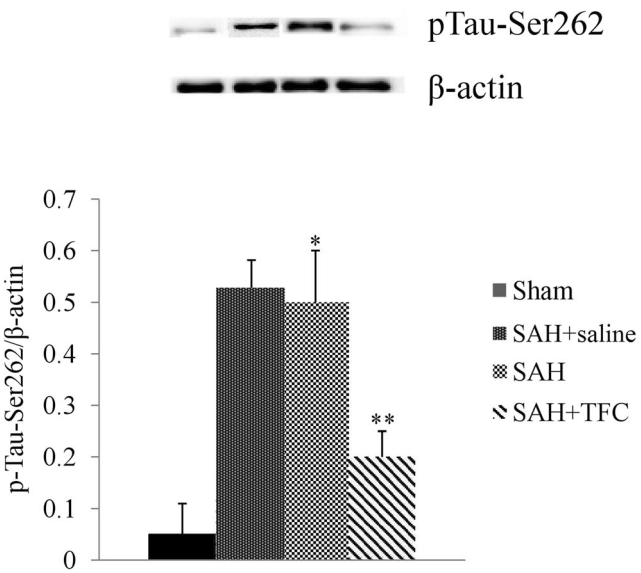

Fig. 6 Effect of TFC onTau-Ser262 phosphorylation (mean ± SD, n = 04), *p < 0.01,vs. Sham, **p < 0.01 SAH

We next sought to investigate the mechanism of protective effect of TFC on EBI after SAH using PCR and Western blot to evaluate the Bcl-2 and Bax expressions. As has previously been reported, apoptosis was considered as one of the major factors that could cause EBI after SAH (Hou et al. 2017; Sabri et al. 2008). Bcl-2 family proteins have crucial role in controlling the apoptosome-mediated pathway, which is one of two genetically regulated biological processes of apoptosis. The proapoptotic (Bax) and antiapoptotic (Bcl-2) proteins may turn on or off apoptosis, the ratio of the expression of the Bax and Bcl-2 is important for cell death and survival (Anto et al. 2002; Ghate et al. 2014).In present study, we found that the SAHinduced remarkable up-regulation of the Bax/Bcl-2 ratio both in mRNA and protein levels, which could be ameliorated by TFC administration. These findings suggested that the protective role of TFC in EBI after SAH may be related to protect neuron from apoptosis.

To further confirm the protective effect of TFC on delay cognitive dysfunction, the step down test and MWM were used to assess spatial learning memory ability (Wen et al. 2019). In agreement with the previous results, we found that experimental SAH led to a significant learning and memory dysfunction, and could be inhibited by TFC treatment.

Tau, one of microtubule-associated proteins, is normally enriched in neuronal axons, and maintains the microtubule stability. However, highly phosphorylated tau protein will be detached from microtubules and sequentially aggregated into oligomers, protofibrils and fibrils, and ultimately leads to the formation of insoluble neurofibrillary tangles (NFTs) in the cytoplasm. NFTs are closely related to variously cerebral pathologies after injury, including SAH, and directly correlated with cognitive deficits (Joswig et al. 2018). Neuropsychological deficits induced by SAH correlates with increased total Tau and phosphorylation Tau concentrations in cerebrospinal fluid (Joswig et al. 2018). In particular, the hyperphosphorylation of tau at ser262 directly affects the binding of tau protein to microtubule, and finally depolymerization of microtubule (Fischer et al. 2009). Thus, tau-Ser262 phosphorylation in hippocampal tissue was detected in present study to further evaluate the protection of TFC on delayed injury of SAH. Although the increase of tau-Ser262 phosphorylation after SAH inhibited

by TFC treatment was detected, further studies are needed to firstly determine the mechanism of down-regulation of tau phosphorylation of TFC.

Conclusions

In summary, TFC treatment could inhibit the development of EBI induced by SAH through multiple mechanisms, such as reducing lipid peroxidation, downregulating the Bax/Bcl-2-related apoptosis pathway, and then ameliorate followingly cognitive dysfunction which was related with its inhibition of tau phosphorylation. Of course, further studies are of crucial importance to elucidate the other inhibitory phosphorylation site of TFC at tau protein.

Authors' contribution Wen JY: Participated in research design and writing of the manuscript; Lu WZ: Participated in experiments and data analysis.

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Compliance with ethical standards

All animal experiments were reviewed and approved by the Ethics Review Committee of Anhui Medical University, which comply with the Guide for the Care and Use of laboratory Animals published by the US National Institutes of Health (NIH publication no. 85 − 23, revised 2011).

Conflict of interest The authors have no conflicts of interest to disclose.

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