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ORIGINAL ARTICLE

The Role of Total Flavone of *Camellia* on Cerebrovascular Vasopasm after Subarachnoid Hemorrhage in Rats

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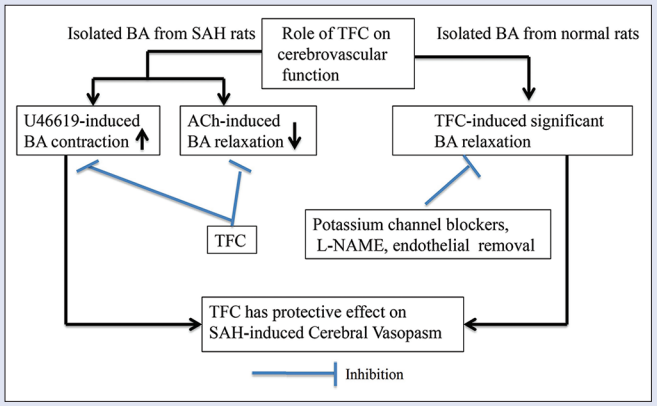
ABSTRACT

Background: This study was undertaken to explore the role of total flavones of *Camellia* (TFC) on the cerebrovascular dysfunction in rats after experimental subarachnoid hemorrhage (SAH). **Materials and Methods:** The contraction and dilation of cerebral basilar artery (BA), nitric oxide (NO) level, and nitric oxide synthase (NOS) activity in rat serum and the expression of endothelial NOS (eNOS) in cerebral vessels were measured. Furthermore, the effect of potassium channel blockers, endothelium removal, L-NG-nitroarginine methyl ester (L-NAME), or prostacyclin I_2 (PG I_2) production inhibitor on the response of isolated BA derived from normal rats was also evaluated to explore the underlying mechanism of vasodilation induced by total flavones. **Results:** The contraction of rat BA to U46619 markedly increased and the vasodilation to acetylcholine remarkably reduced after SAH. Interestingly, these vascular dysfunctions were profoundly ameliorated by pretreatment of TFC. Moreover, total flavones could induce a concentration-dependent relaxation in isolated BA from normal rats, which was obviously eliminated by co-application of potassium channel blockers, ChTx and Apamin, application of L-NAME, or endothelial removal. In addition, total flavones pretreatment obviously improved the expression of eNOS in BA, serum NO level, and NOS activity at 48 h after SAH. **Conclusion:** These findings revealed that TFC has protective effect on cerebrovascular dysfunction after SAH and demonstrated that the protection could be due to its upregulation of eNOS expression and activation of potassium channel.

Key words: Basilar artery, endothelial nitric oxide synthase, subarachnoid hemorrhage, total flavones of *Camellia*, vasospasm

SUMMARY

- In this study, the role of total flavones of *Camellia* (TFC) on the cerebrovascular dysfunction in rats after experimental subarachnoid hemorrhage (SAH) was investigated. The results revealed that TFC has protective effect on cerebrovascular dysfunction induced by experimental SAH; the protection could be due to its upregulation of endothelial nitric oxide synthase expression and activation of potassium channel.



Abbreviations used: TFC: Total flavones of *Camellia*; SAH: Subarachnoid hemorrhage; BA: Basilar artery; NO: Nitric oxide; NOS: Nitric oxide synthase; L-NAME: L-NG-Nitroarginine methyl ester; PG I_2: Prostacyclin I_2; CV: Cerebral vasospasm; CJ: *Camellia japonica* L.; ECJ: Extract of *Camellia japonica* L. flower.

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INTRODUCTION

Cerebral vasospasm (CV) is one of the most common and debilitating neurological complications of subarachnoid hemorrhage (SAH) and is one of the leading causes of morbidity and mortality in SAH patients.[1] Despite numerous experiments and clinical studies, proven therapeutic options for the prevention or treatment of CV are few.[2] The current treatment recommendations for vasospasm could be as summarized as follows: prophylactic nimodipine; directed application of vasodilating agents or a potential for balloon angioplasty; hypervolemia with induced hypertention.[3] However, the overall outcome of SAH patients have not yet improved,[4] the lack of clear effects of the smooth muscle relaxants is considered as an important reason for unconspicuous improvement of outcome of SAH patients.[5] Therefore, effective treatment for CV depends on the directly cerebral vasodilation, and vascular protection is regarded as an importantly therapeutic approach to reduce SAH damage.[6]

Camellia japonica L. (CJ) is a member of the tea family and widely cultivated in China and other parts of the world. Previous studies have reported that CJ possesses a variety of bioactivities, such as cerebral protection and endothelium-dependent vasodilation.[7,8] We also found in previous studies that the extract of CJ flower (ECJ) has protective effect on hippocampal neurons following anoxia/reoxygenation injury[9] and early brain injury at 48 h after SAH (unpublished). TFC are the effective parts of ECJ, whether ECJ or TFC can safely exert protective role on cerebrovascular dysfunction after SAH is still unknown. Thus, the present research was designed to demonstrate the potential effect

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of TFC on cerebrovacular dysfunction following SAH and explore its underlying mechanism.

MATERIALS AND METHODS

Drugs and reagents

The TFC was purchased from Xi'an Wanfang Biological Technology Co., Ltd (Xi'an, China). Nitric oxide (NO) and nitric oxide synthase (NOS) test kits were purchased from Nanjing Jiancheng Bilolgical Company (Nanjing, China).

Subarachnoid hemorrhage model

Adult Sprague–Dawley rats (male to female: 1:1), weighing 220–250 g, were purchased from Anhui Medical University Animal Center and housed and habituated to the novel environment for 1 week under standard laboratory conditions. All experimental procedures were approved by the Ethics Review Committee of Hefei Technical College, which conforms to 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). Sixty rats were randomly divided into six groups: SAH group, sham group, sham + TFC (80 mg/kg) group, SAH + TFC (80 mg/kg) group, SAH + TFC (40 mg/kg) group, and SAH + TFC (20 mg/kg) group. TFC or equal volume of saline was administrated through gavage once a day for 5 consecutive days.

Experimental procedures of rat SAH was induced using single-hemorrhage model under anesthesia (350 mg/kg chloral hydrate).[10] Briefly, 200 μL autologous blood was withdrawn from the femoral artery and then injected into the cisterna magna over a 3-min period. At 48 h after blood injection, the rats were sacrificed under anesthesia to obtain the whole brain, isolated basilar artery (BA), and serum samples.

Measurement of serum nitric oxide level and nitric oxide synthase activity

Briefly, rat sera were collected and transferred to 96-well plates, respectively, for NO level and NOS activity determination using biochemistry assay kit (Jiancheng Bioengineering Ltd., Nanjing, China) and abiding by the manufacturer's manual.

Vessel experiment

As described previously,[11] the BA was isolated from rat at 48 h after SAH or normal rat, placed in precooled Krebs solution and cut into serial segments of 3-mm length. Subsequently, segment of vessel was secured with a nylon monofilament suture, placed in a perfusion chamber, equilibrated with Krebs solution (at 37°C, continuously aerated with a gas mixture of 5% CO_2 and 95% O_2), and then pressurized to 85 mmHg. Where after, the luminal flow was adjusted to 150 μl/min, at 60 min after equilibrium, 1×10^{-7} mol/l U46619 (final concentration) was added to the luminal perfusate to obtain a stable contraction. The tension of the artery was continually measured utilizing myograph. The maximum rate of vascular dilation of BA was calculated by following formula: Dilation (%) = $(T_{min} - T_x)/(T_{min} - T_{max}) \times 100\%$, where T_{min} is the stably tension of artery precontracted with U46619, T_x is the vascular tension after administration of TFC or Vehicle, and T_{max} is the initially vascular tension.

Western blot

The total proteins in cerebral BA were electrophoresed in a 12% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h in Tris-buffer saline containing skim milk in and then incubated overnight at 4°C in the same buffer with 1:1000 dilution of anti-endothelial nitric oxide synthase (eNOS) (1:500, Cell Signaling Technology, USA) or 1:2000 dilution of the antibody of β-actin (1:2000; Sigma-Aldrich, USA) used as an internal control. After six washes of 10 min each, a horseradish peroxidase-conjugated anti-rat IgG (KPL, Gaithersburg, MD, USA) was added at a dilution of 1:2000 in buffer with 5% skim milk. The relative intensity of the band was determined by densitometry.

Statistical analysis

One-way analysis of variance followed by the Tukey's test was performed to determine the difference between groups, $P < 0.05$ is considered statistically significant. Isolated blood vessel data are presented as mean ± standard deviation, while the other data are expressed as mean ± standard error of the mean.

RESULTS

Effect of total flavones of *Camellia* on the cerebovascular dysfunction of rats injured by subarachnoid hemorrhage

The changes of vascular tension in rat BA were examined at 48 h after SAH. The results were shown in Figure 1, in sham group, 10^{-7} mmol/L U46619 evoked significant constriction of BA with maximum possible effect (E_{max}) of 1.56 ± 0.33 mN. However, the BA contraction in SAH rats to U46619 profoundly elevated and the E_{max} being increased to 2.35 ± 0.24 mN. Interestingly, the increment of BA contraction was

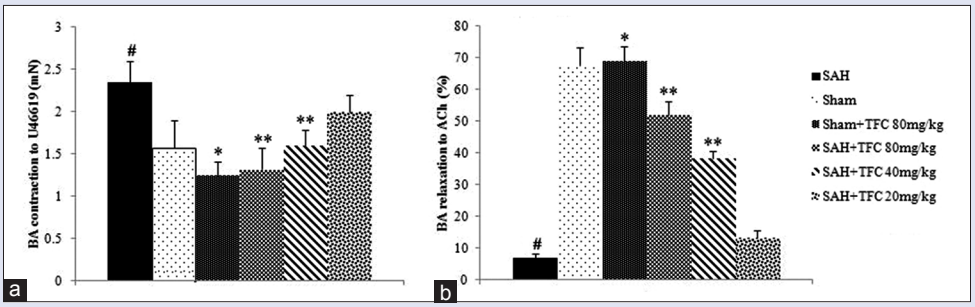

Figure 1: Total flavones of *Camellia* attenuated cerebrovascular dysfunction induced by subarachnoid hemorrhage in rats (mean ± standard deviation, $n = 8$). (a) The effect of total flavones of *Camellia* on U46619-meditated basilar artery contraction. (b) The effect of total flavones of *Camellia* on acetylcholine-meditated relaxation in basilar artery preconstricted with U46619. #$P < 0.01$ versus sham, **$P < 0.01$ versus subarachnoid hemorrhage, *$P > 0.05$ versus Sham

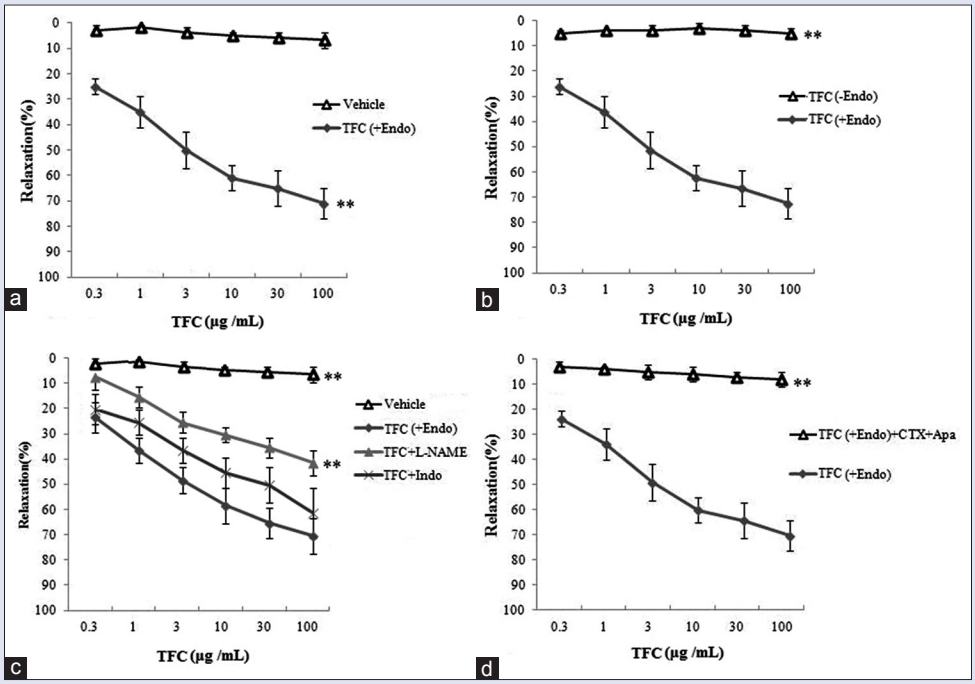

Figure 2: Total flavones of *Camellia*-mediated relaxation of basilar artery from normal rats (mean ± standard deviation, $n = 8$). (a) Effect of total flavones of *Camellia* on relaxation in U46619-preconstricted basilar artery. $^{**}P < 0.01$ versus Vehicle. (b) Effect of the endothelium removal on the total flavones of *Camellia* -induced relaxation. $^{**}P < 0.01$ versus total flavones of *Camellia* (+Endo). (c) Effects of L-NG-nitroarginine methyl ester, Indo on the total flavones of *Camellia*-mediated relaxation in basilar artery. $^{**}P < 0.01$ versus total flavones of *Camellia* (+Endo). (d) Effects of K_{Ca} channel blockers CTX and Apa on total flavones of *Camellia*-mediated relaxation in BA. $^{**}P < 0.01$ versus total flavones of *Camellia* (+Endo)

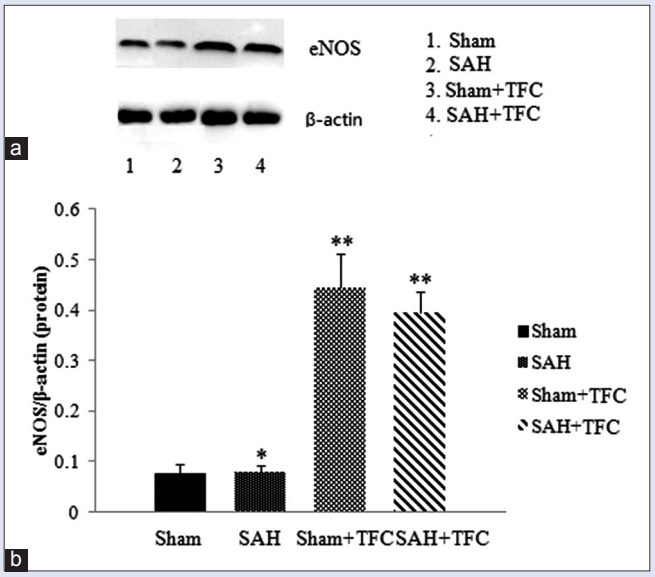

Figure 3: Effect of total flavones of *Camellia* on endothelial nitric oxide synthase expression in basilar artery (Western blot method, mean ± standard error of the mean, $n = 4$). (a) Expression of endothelial nitric oxide synthase. (b) Endothelial nitric oxide synthase was quantified by calculating the densitometric ratio of the bands of endothelial nitric oxide synthase to β-actin. $^{**}P < 0.01$ versus subarachnoid hemorrhage, $^{*}P > 0.05$ versus sham

significantly reduced by 40 or 80 mg/kg TFC pretreatment. Moreover, the acetylcholine (Ach)-mediated relaxations of BA were also remarkably inhibited by SAH, the E_{max} being reduced from 66.67% ± 5.64% of sham group rat to 6.83% ±1.47% of SAH group rat; the vasodialation dysfunction in BA after SAH was also ameliorated by 40 or 80 mg/kg TFC pretreatment.

Total flavones of *Camellia* -mediated relaxation of basilar artery

We then aimed to demonstrate the effect of TFC on isolated BA of normal rat and further explore the underlying mechanism. As shown in Figure 2a, in the range of 0.3–100 μg/mL, TFC could induce remarked dilation of BA from normal rat to U46619 (E_{max}: 71% ±8%), but the relaxation of BA was completely blocked when we removed the endothelium, E_{max} being reduced to 5% ±0.5% [Figure 2b]. These results indicated that the dilation of BA to TFC is endothelium-dependent. Moreover, Figure 2c shows that the NOS inhibitor L-NG-nitroarginine methyl ester (L-NAME) (30 μmol/L) markedly inhibited the TFC-induced BA dilation. But, the Indo (10 μmol/L) had not obviously role on TFC-induced vasodilation (compared to TFC + endo group, $P > 0.05$), suggesting that NO but not PGI_2 involved in the TFC-induced vasodilation BA dilation. Furthermore, the relaxation of BA to TFC was also obviously abolished by co-application of ChTx (CTX) and Apamin (Apa), E_{max} being reduced from 71% ± 8% to 8% ± 1.0% [Figure 2d]. These data suggested that K_{Ca} channel might involve in the TFC-induced BA dilation.

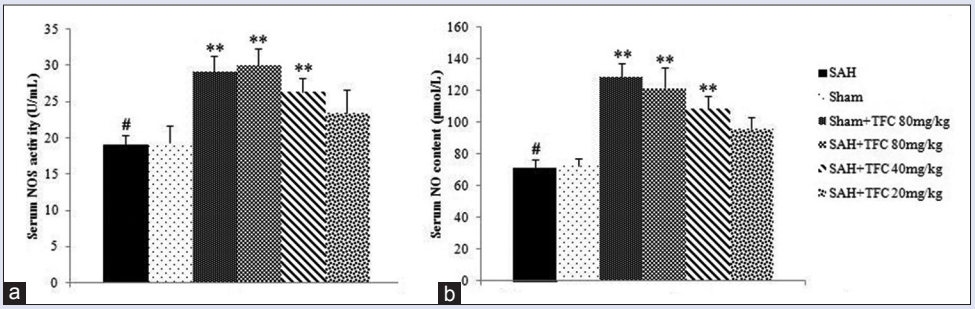

Figure 4: Effect of total flavones of *Camellia* on rat serum nitric oxide synthase activity and nitric oxide content (mean ± subarachnoid hemorrhage, $n = 8$). (a) Serum nitric oxide synthase activity. (b) Serum nitric oxide level. $^{\#}P > 0.05$ versus Sham; $^{**}P < 0.01$ versus subarachnoid hemorrhage

Effect of total flavones of *Camellia* on the expression of endothelial nitric oxide NOS in cerebral basilar artery after subarachnoid hemorrhage

The expression of eNOS in BA was detected using Western blot. As shown in Figure 3, the eNOS expression increased remarkably in TFC groups (sham + TFC and SAH + TFC). These result indicated that the upregulation of eNOS might also involve in the TFC-induced cerebrodilation.

Effect of total flavones of *Camellia* on rat serum nitric oxide level and endothelial nitric oxide NOS activity at 48 h after subarachnoid hemorrhage

We next sought to determine the NO level and eNOS activity in rat serum after SAH. As shown in Figure 4, 40 or 80 mg/kg TFC pretreatment remarkably elevated the serum NO level and eNOS activity. The changes of serum NO level and eNOS activity were in agreement with the altered eNOS expression in cerebral BA. These findings support the aforementioned result that TFC-induced cerebrovasculaion may relate to upregulation of eNOS.

DISCUSSION

CV is one of the main causes of morbidity and mortality after SAH, even when the surgical clipping of aneurysm or the endovascular coil embolism is successful.[12,13] Previous study has reported that CV was considered as the primary marker to monitor patient' progression.[14] The severity and extent of the ischemia-induced cerebral injury following vasospasm are responsible for the neurological deficits and overall poor outcome in such cases.[15] The smooth muscle relaxants, such as endothelin receptor antagonists, Rho-kinase inhibitors, and calcium antagonists, have been used for the treatment of vasospasm, but the overall outcome of SAH patients has not significantly improved.[4] Therefore, the main priority is to explore the therapies for vasospasm after SAH.

Previous studies have reported that CJ possesses bioactivity of endothelium-dependent vasodilation.[7,8] The present study revealed that the contraction of BA to U46619 profoundly increased after SAH, and the increment of the contraction was significantly ameliorated by 40 or 80 mg/kg TFC supplement. However, the dilation of BA to ACh was remarkably reduced after SAH; not surprisingly, the reduction of BA dilation to ACh was also obviously ameliorated by 40 or 80 mg/kg TFC pretreatment. These data indicated that TFC had significant protection on cerebrovascular dysfunction induced by SAH.

We next sought to demonstrate the mechanism of BA relaxation to TFC using NO synthase inhibitor L-NAME, PGI_2 production inhibitor indomethacin, K_{Ca} channel blockers, and endothelial removal. We found that relaxations of isolated BA from normal rat to TFC were obviously abolished by LAME, co-application of the small-conductance K_{Ca} channel blocker Apa, and intermediate-conductance K_{Ca} channel blocker CTX, or endothelial removal. However, the Indo had not obviously role on TFC-induced vasodilation. These results provided solid evidence that the vasodilation of cerebral vessels to TFC is endothelium-dependent and might relate to upregulation of the NO synthesis and activating the K_{Ca} channel.

NO plays a significant role in regulating hemodynamic and vascular activity; it dilates blood vessels and inhibits leukocyte adhesion to the endothelial layer and platelet aggregation. Endogenous NO is produced by NOS, which contain eNOS, inducible NOS NOS, and neuronal NOS.[16] Alterations in NO pathways have been demonstrated in the early period after SAH both in humans and animals.[17,18] To further confirm the relationship between the TFC-induced vasodilation with upregulation of NO, the eNOS expression in rats cerebral vessels, the NO level and eNOS activity in rat serum were detected at 48 h after SAH. The results revealed that TFC pretreatment significantly elevated the rat serum NO level and eNOS activity and increased the expressions of eNOS protein in rat BA. These findings gave us more detail and demonstrating relationship between the TFC-induced vasodilation with upregulation of NO.

CONCLUSION

Despite the great progress in surgical techniques, the outcome of patients with a SAH remains unsatisfactory, cerebral vasospasm is one of main causes of morbidity and mortality after SAH. Our study is the first showing the multifaceted vasoprotection of TFC on SAH injury. We found that (1) TFC had eminently protective role on vasomotor dysfunction induced by SAH in rats; (2) K_{Ca} channel might involve in the cerebral vasodialtion to TFC; (3) the cerebrovascular dilation to TFC is endothelium-dependent and closely related to upregulation of eNOS expression.

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Conflicts of interest

There are no conflicts of interest.

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