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Protective mechanisms of tea polyphenols regulating the PI3K/Akt pathway on early brain injury after subarachnoid hemorrhage in rats

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ABSTRACT

In recent years, numerous studies have demonstrated that tea polyphenols (TPPs) can exert neuroprotective effects through the regulation of the PI3K/Akt pathway. The objective of this work was to verify whether TPPs could protect against early brain injury in rats after subarachnoid hemorrhage (SAH) by modulating the PI3K/Akt pathway. A total of 150 rats were randomly rolled into control (C), TPP, and SAH groups. The TPP and SAH groups underwent endovascular perforation to induce SAH, while C group received only endovascular needle puncture and saline injection. Brain water content, Evans Blue (EB) extravasation assay, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, Western blot, and RT-PCR analyses were performed. Relative to SAH group, TPP treatment considerably improved neurological function scores following SAH, reduced brain edema, cortical neuronal apoptosis, and blood-brain barrier damage. Levels of aquaporin-4 (AQP4) and apoptosis-related protein Bax were considerably lower in the TPP group than in SAH group. Conversely, levels of anti-apoptotic protein Bcl-2 and tight junction protein Zona occludens 1 (ZO-1) were considerably higher in the TPP group. Furthermore, TPP treatment was found to activate the PI3K/Akt signaling. TPPs can mitigate early brain injury caused by SAH in rats by reducing AQP4 levels, alleviating cortical damage, and attenuating neuronal apoptosis. These findings elucidate the protective mechanisms of TPPs against early brain injury following SAH through the regulation of the PI3K/Akt signaling.

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Introduction

Subarachnoid hemorrhage (SAH) is a common neurological emergency with an incidence rate of approximately 10 per 100,000 population (1). The primary etiologies of SAH include ruptured cerebral aneurysms, arteriosclerosis, and intracranial arterial malformations (2). Early brain injury following SAH is the leading cause of mortality and disability in patients, involving multiple factors such as neuronal apoptosis, inflammatory responses, oxidative stress, and neuronal-glial cell imbalance (3). Currently, the treatment strategies for SAH mainly consist of surgical intervention and pharmacotherapy. Nevertheless, there is currently no specific drug available for effectively treating early brain injury in SAH (4). Hence, a comprehensive approach to SAH treatment is required, encompassing early neuroprotection, inflammation control, oxidative stress inhibition, and regulation of neuronal-glial cell balance. Additionally, individualized treatment plans tailored to the specific etiology of SAH are necessary to achieve optimal therapeutic outcomes. In the future, with further in-depth research into the pathogenesis of SAH and the continuous development of novel therapeutic approaches, it is anticipated that more hope and opportunities will emerge for the treatment of SAH.

Tea polyphenols (TPPs) are natural polyphenolic compounds that contain multiple hydroxyl groups in their molecular structure. They possess antioxidant, anti-inflammatory, and neuroprotective properties and have been widely utilized in the treatment of cardiovascular diseases, tumors, and neurological disorders (5,6). The neuroprotective effects of TPPs have been extensively studied and applied (7). In the treatment of neurological disorders, TPPs can alleviate neuroinflammatory responses, and promote neuronal growth and regeneration, thereby improving neurological functions (8).

The PI3K/Akt pathway is a crucial cellular signaling involved in the regulation of various biological processes, including cell proliferation, survival, metabolism, and motility (9). The main components of this pathway include phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), and phosphatase and tensin homolog (PTEN). When cells are stimulated by external factors, PI3K is activated and converts phosphatidylinositol into phosphatidylinositol 3,4,5-trisphosphate (PIP3), which in turn activates Akt. Akt further regulates multiple downstream signaling molecules, such as mTOR, GSK-3β, and FOXO, thereby influencing cellular behaviors. The PI3K/Akt pathway plays a crucial role in various diseases, including cancer, diabetes, and cardiovascular diseases, making it a crucial target for drug development (10,11).

In recent years, an increasing number of studies have demonstrated that TPPs can exert their neuroprotective effects by modulating the PI3K/Akt pathway. Nevertheless, there is limited mention of the protective effects of TPPs on early brain injury after SAH through the regulation of the PI3K/Akt pathway. Hence, this work aimed to verify whether TPPs can protect against early brain injury in rats

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after SAH by modulating the PI3K/Akt pathway.

Materials and Methods

Experimental animals

All experimental animals were purchased from the Animal Laboratory of the Third People's Hospital of Chengdu University. The animals utilized in the experiments were adult male Sprague-Dawley rats, 10 weeks old, with a body weight ranging from 280 to 320 grams. The rats had ad libitum access to standard rodent feed and water. The experimental procedures in this work were approved by the Third People's Hospital of Chengdu Ethics Committee and strictly adhered to the guidelines for the handling and care of laboratory animals set forth by the National Institutes of Health in the United States.

Experimental design

In this experiment, a total of 150 rats from the same batch were assigned into three groups randomly: control (C) group, TPP group, and SAH group, with 50 rats in each group. Based on relevant studies (12,13), the rats in the TPP group and SAH group underwent intravascular puncture to induce SAH, while the rats in the C group received vascular puncture without SAH induction and were injected with an equal volume of physiological saline. During the experiment, C group and SAH group rats were provided with the same standard rodent feed, while the TPP group received rodent feed enriched with TPPs. Three days after the surgery, all rats underwent behavioral testing, and euthanasia was performed on the rats three days after the surgery. For each group, 10 rats were utilized for measuring brain water content, 10 rats underwent Evans blue (EB) extravasation experiment to assess blood-brain barrier function, 10 rats were utilized for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and 20 rats were utilized for Western blot and RT-PCR analysis.

SAH model construction

SAH refers to a common cerebrovascular disease in which blood enters the subarachnoid space due to the rupture of blood vessels in the subarachnoid space or ruptured vascular malformations, resulting in pathological changes such as compression and ischemia in the brain tissue. To gain a deeper understanding of the pathogenesis of SAH and identify effective treatment methodologies, researchers often adopt animal models for experimental studies. These animal models simulate the pathological processes of human SAH, allowing for investigations into the pathophysiology, molecular biology, neuroimaging, and other aspects of SAH, and providing an imperative reference for clinical treatment. Based on methodologies described in other experiments, the SAH model in rats was established through intravascular puncture (Table 1).

Detection of brain water content

To assess the extent of brain edema, the wet/dry weight methodology was employed. Firstly, the rat brains were carefully removed and immersed in a physiological saline solution for 30 minutes. Subsequently, three weighings were performed, and the average was taken to determine the wet weight (accurate to 0.1 milligrams). Next, the brain tissue was placed in a drying oven for dehydration to obtain the dry weight. Finally, the percentage of brain water content in rats was calculated as follows: brain water content = (wet weight - dry weight) / wet weight × 100%. Through this methodology, the degree of brain edema in rats can be accurately evaluated.

EB staining

On the third day following the surgical procedure in rats, anesthesia was administered for experimental purposes. While under anesthesia, EB dye was injected into the left femoral vein to track its distribution in subsequent experiments. After two hours, the rats were re-anesthetized to induce a deep anesthesia state. The heart was then exposed by sternotomy to initiate cardiac perfusion, ensuring proper cardiac function. Once perfusion was completed, the right brain tissue was extracted and weighed, followed by homogenization. The homogenate was subsequently centrifuged, and the supernatant was collected to measure the content of EB dye, thereby determining its distribution in the brain tissue. This approach ensured the accuracy of the experimental results.

Tubular paraffin section of rat brain tissue

After induction of deep anesthesia, the thorax of the rats was incised to adequately expose the heart. Subsequently, a perfusion solution was applied to flush the rat's heart, while simultaneously cutting the right atrium for blood drainage. To ensure successful perfusion, the vigorous twitching of the limbs was observed until rigor mortis occurred in the limbs and neck, and the brain tissue exhibited a firm and white appearance. Once perfusion was completed, the posterior neck muscles were separated and removed using tissue scissors, followed by careful removal of the skull with forceps to extract the brain tissue. Finally, the rat's brain tissue was placed in a brain mold for fixation and subjected to embedding, sectioning, and other processing procedures for subsequent experimental studies. This process requires utmost care to ensure the integrity and accuracy of the brain tissue. Additionally, adherence to relevant ethical regulations is crucial to safeguard the welfare and rights of the animals.

Table 1. Construction steps of rat endovascular perforation model.

No.	Intravascular perforation procedure
1	Anesthetize rats and fix them on the operating table
2	Clean the surgical site with disinfectant and disinfect it
3	Puncture the carotid artery of rats with a puncture needle and insert the catheter into the artery
4	Connect the syringe to the catheter and inject blood or blood substitute, so that the blood or blood substitute can enter the cerebral vessels
5	Wait a while for blood or blood substitutes to form hematoma in the cerebral vessels

Table 2. TUNEL staining steps.

No.	TUNEL staining operation steps
1	Paraffin tissue slices were dewaxed
2	The samples after dewaxing were repaired
3	The samples were washed three times for 5 minutes each time, and then kept in a wet box
4	Each sample was dripped with 50 microliters of Equilibration Buffer and incubated at room temperature for 10 minutes
5	Enough TdT incubation buffer was prepared
6	After the residual liquid was removed from the sample, the incubation buffer was added and incubated at 37 degrees Celsius for 1 hour in the dark
7	After washing with PBS for three times, the nucleus was stained again
8	The film was sealed with sealing liquid containing anti-fluorescence quencher, and then the collected images were observed under fluorescence microscope
9	TUNEL-positive neurons were green under fluorescence microscope, while TUNEL-negative nuclei were blue under DAPI staining

Table 3. PCR operation steps.

No.	PCR operation steps
1	Preparation of DNA template: DNA was extracted from the sample and purified.
2	Preparation of reaction system: the reagents needed for PCR reaction were mixed according to a certain proportion, including template DNA, primers, dNTPs, enzyme and buffer.
3	Thermal cycling program: PCR reaction needs many thermal cycles, each cycle includes thermal denaturation, primer binding, and DNA synthesis.
4	PCR product analysis: PCR products were analyzed and detected by electrophoresis and other methodologies.
5	Results interpretation: according to the quantity and size of PCR products, whether the target DNA sequence existed or not can be judged.

TUNEL staining

According to Table 2, the following steps were followed to perform TUNEL staining on paraffin-embedded sections of rat brain tissue.

Western blot (WB) detection of protein expression

WB is a widely employed protein detection technique in biological research. Its fundamental principle involves the separation of the protein samples of interest through electrophoresis, followed by their transfer onto a polyacrylamide gel. The presence and expression levels of the target protein are then detected by specific antibody binding (14). WB offers high sensitivity, strong specificity, and ease of use, enabling the detection and quantitative analysis of single or multiple proteins.

The procedure involved the dissection of rat brains, followed by homogenization of the tissue to create a tissue lysate. The lysate was then centrifuged, and the supernatant was collected. The lysate was supplemented with lysis buffer in a ratio of 10 mg/μL to prepare the samples for analysis. The samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), separating the proteins into distinct bands regarding their molecular weight. The separated proteins were transferred onto a polyacrylamide gel, commonly using a semi-dry or wet transfer method. The transferred gel was then blocked to prevent nonspecific binding. The target protein was detected by binding it with specific antibodies, typically using a primary and secondary antibody combination. The presence and levels of the target protein were detected using secondary antibodies labeled with markers such as horseradish peroxidase or alkaline phosphatase.

RT qPCR detection

PCR is a technique used for detecting DNA or RNA (15). It amplifies specific sequences of the target DNA or RNA, increasing their quantity to a detectable level. PCR detection finds applications in disease diagnosis, pathogen detection, and genotyping, among others. RNA extraction from rat brain tissue was performed using a specific method, and the concentration of RNA was measured using a spectrophotometer. Subsequently, the RNA was transcribed into complementary DNA (cDNA) using reverse transcriptase. This process facilitates the study of gene expression and regulatory mechanisms in rat brain tissue. The specific operational steps are outlined in Table 3.

Statistical evaluation

Data were processed using Excel, and the results were expressed as mean±SD ($\bar{x}\pm s$). $P<0.05$ was considered statistically significant.

Results

Comparison of mortality and SAH grade

In the C group, all rats survived, while in the SAH group, 20% of the rats died. In the TPP group, the mortality rate of rats was 19%. To ensure an equal number of experimental animals, the study excluded dead and disqualified animals and supplemented them with new rats. Additionally, the relevant experimenters performed SAH grading evaluations on the rats, and the results showed a slight difference in SAH grades between the TPP group and the SAH group ($P>0.05$).

TPPs alleviated early brain injury in rats

Three days after the surgery, early brain damage was assessed using neurobehavioral scoring, brain edema, and blood-brain barrier integrity. The scores for each parame-

Table 4. Comparison of data of different items of rats in each group.

Item	Group		
	C group	SAH group	TPP group
Evans blue permeability	1.9	5.78*	3.77#
Water content	78.67	81.1*	79.57#
Balance beam test score	3.7	1.6*	2.6#
Garcia scoring point	17.6	13.67*	15.32#
TUENM neuron positive rate (%)	7.67	45*	33.5#

Note: *P<0.05 SAH group vs. C group; #P<0.05 TPP group vs. SAH group (* and # had the same meanings for all figures below).

ter are presented in Table 4.

Effect of TPPs on compact protein

In maintaining the integrity of the blood-brain barrier, Zona occludens 1 (ZO-1) and claudin-5 play crucial roles as transmembrane proteins. The expression levels of tight junction-associated proteins were assessed using WB, and it was observed that after SAH, the protein levels of ZO-1 and claudin-5 considerably decreased (P<0.05). Nevertheless, following treatment with polyphenols, the TPP group showed a notable improvement in the protein levels of ZO-1 and claudin-5 relative to the SAH group (Figure 1).

TPPs decreased the expression of aquaporin-4 (AQP4)

The AQP4 protein level in the cortical region was assessed using WB. In Figure 2, 72 hours after the surgery, there was a drastic upregulation of AQP4 protein levels (P<0.05). Nevertheless, following intervention with polyphenols, the TPP group showed a notable decrease in AQP4 protein (P<0.05). Furthermore, AQP4 mRNA levels in the cortical region were assessed using RT-PCR. The results indicated that after SAH, a marked increase was suggested in AQP4 mRNA levels. Nevertheless, in the TPP group, following treatment with polyphenols, there was a remarkable reduction in AQP4 mRNA levels (P<0.05).

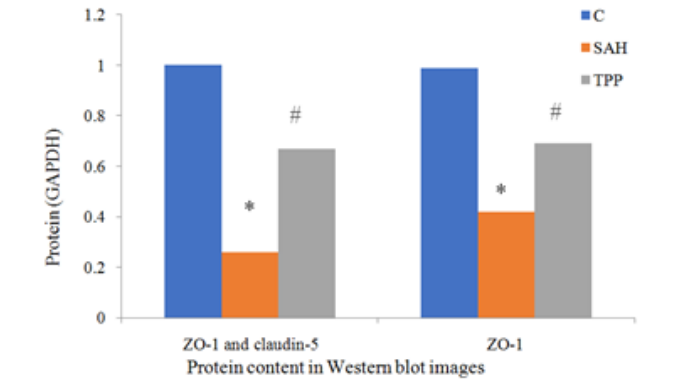

Figure 1. Effect of TPPs on tight junction protein level in the cerebral cortex of rats with SAH.

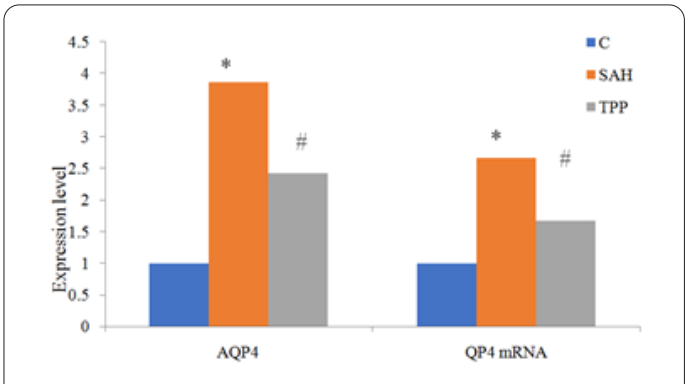

Figure 2. Effect of TPPs on AQP4 level in the cerebral cortex of rats with SAH.

TPPs activated P13K/Akt signaling

In Figure 3, relative to the C group, levels of p-PI3K and p-Akt protein were considerably decreased in the SAH group (P<0.05). Nevertheless, consumption of a diet rich in polyphenols led to an increase in these proteins (P<0.05). Hence, polyphenols have the ability to activate the PI3K/Akt signaling and restore the phosphorylation status of the pathway.

Effect of TPPs on apoptosis protein

Apoptosis-related proteins in the rat cortical region were evaluated using WB. From Figure 4, following SAH, the expression of the pro-apoptotic protein Bax considerably increased in the SAH group relative to the C group, while that of the anti-apoptotic protein Bcl-2 decreased

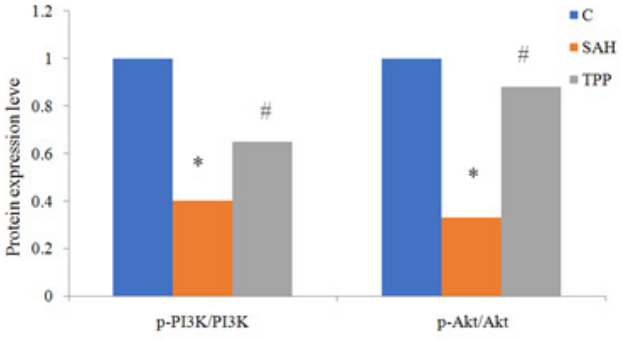

Figure 3. Effect of TPPs on PI3K/Akt pathway.

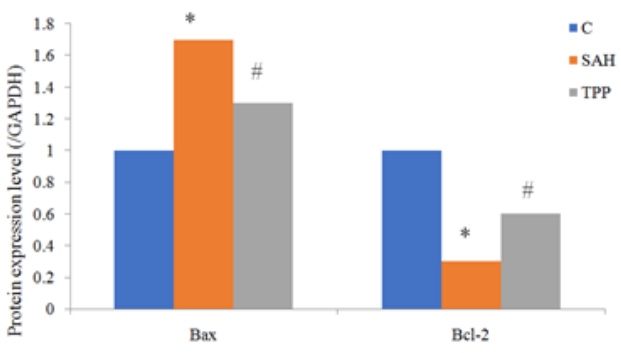

Figure 4. Effect of TPPs on apoptosis-related proteins in the cerebral cortex of rats with SAH.

considerably (P<0.05). Nevertheless, after intervention with polyphenols, the TPP group showed a great decrease in Bax protein relative to the SAH group, and a marked increase in the Bcl-2 protein relative to the SAH group (P<0.05).

Discussion

SAH is a common brain disorder caused by the rupture of blood vessels within the subarachnoid space of the brain. This condition leads to the leakage of blood into the subarachnoid space, resulting in severe damage to the nervous system (16). Patients may experience symptoms such as headache, nausea, vomiting, and impaired consciousness, and in severe cases, seizures and coma may occur. Additionally, SAH can cause complications such as brain edema, cerebral vasospasm, and hydrocephalus, imposing significant suffering and danger to the patients (17). Brain edema serves as a crucial indicator for early brain injury detection and is an independent risk factor for mortality and adverse reactions (18). In this experiment, it was observed that the water content in the brain tissue of SAH rats was superior to that in the C group, while the TPP group exhibited lower water content than the SAH group. This finding demonstrates that SAH can induce brain edema, while polyphenols can reduce SAH-induced brain edema and mitigate cellular apoptosis resulting from brain edema.

TPPs are natural polyphenolic compounds that possess potent antioxidant effects. They have the ability to scavenge free radicals and alleviate oxidative stress, thereby protecting cells from oxidative damage (19,20). This work demonstrated that TPPs can reduce the expression of AQP4, repair the damage to tight junction proteins in the rat cortical region caused by SAH, and decrease the expression of apoptotic proteins that are increased in the cortical region following SAH. These findings suggest that TPPs can inhibit early neuronal apoptosis and inflammatory responses after SAH, and promote neuronal survival and recovery.

The antioxidant effects of TPPs may be one of the mechanisms through which they protect neurons (21,22). Additionally, TPPs can inhibit inflammatory responses and alleviate neuronal damage. These effects may be associated with the modulation of the PI3K/Akt pathway by TPPs (23). The PI3K/Akt pathway is a crucial signaling involved in various cellular processes such as growth, differentiation, survival, and apoptosis. In the nervous system, the PI3K/Akt pathway also plays a crucial role in promoting neuronal survival and recovery (24,25). This work demonstrated that TPPs can restore the phosphorylation status of PI3K and Akt in the PI3K/Akt pathway, thereby protecting against early brain damage caused by SAH. TPPs, as natural compounds, can protect neurons by modulating the PI3K/Akt pathway, which may be one of the imperative mechanisms underlying their neuroprotective effects (21). The main mechanism of action of TPPs is the activation of the PI3K/Akt pathway to promote neuronal growth and survival, thereby protecting the nervous system from injury and disease.

In the context of studying early brain damage in other SAH models (26,27), the highlight lies in the inclusion of a sham-operated group as a control, allowing for a comparison with the SAH group and the use of TPPs for treating early brain injury. Nevertheless, research on the role of TPPs in modulating the PI3K/Akt pathway in the context of post-SAH brain injury is limited. Furthermore, the use of a single rat strain and a relatively small sample size in this experiment may introduce the possibility of chance factors influencing the results. Hence, future studies should focus on conducting large-scale experiments involving multiple rat strains to ensure the accuracy and reliability of the findings. There are many reports (28-39) about the effect of medicinal plants in the treatment of various diseases.

Conclusion

The results demonstrated the significant therapeutic effects of TPPs on early brain injury caused by SAH. TPPs were found to considerably reduce the overexpression of AQP4, alleviate cortical damage, and attenuate neuronal apoptosis, thereby effectively improving early brain injury in rats following SAH. Additionally, it was observed that TPP treatment increased the phosphorylation levels of PI3K/Akt, suggesting that TPPs may exert their protective effects through partial activation of the PI3K/Akt signaling. These findings provide new evidence and a theoretical basis for the application of TPPs in the treatment of brain injury.

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