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An optimal dose of tea polyphenols protects against global cerebral ischemia/reperfusion injury★

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Abstract

Previous studies addressing the protection of tea polyphenols against cerebral ischemia/reperfusion injury often use focal cerebral ischemia models, and the optimal dose is not unified. In this experiment, a cerebral ischemia/reperfusion injury rat model was established using a modified four-vessel occlusion method. Rats were treated with different doses of tea polyphenols (25, 50, 100, 150, 200 mg/kg) *via* intraperitoneal injection. Results showed that after 2, 6, 12, 24, 48 and 72 hours of reperfusion, peroxide dismutase activity and total antioxidant capacity in brain tissue gradually increased, while malondialdehyde content gradually decreased after tea polyphenol intervention. Tea polyphenols at 200 mg/kg resulted in the most apparent changes. Terminal deoxynucleotidyl transferase-mediated nick end labeling and flow cytometry showed that 200 mg/kg tea polyphenols significantly reduced the number and percentage of apoptotic cells in the hippocampal CA1 region of rats after cerebral ischemia/reperfusion injury. The open field test and elevated plus maze experiments showed that tea polyphenols at 200 mg/kg strengthened exploratory behavior and reduced anxiety of cerebral ischemia/reperfusion injured rats. Experimental findings indicate that tea polyphenols protected rats against cerebral ischemia/reperfusion injury and 200 mg/kg is regarded as the optimal dose.

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Key Words

neural regeneration; brain injury; traditional Chinese medicine; tea polyphenols; brain; ischemia/reperfusion injury; oxidative stress; neuroethology; apoptosis; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) Tea polyphenols have anti-inflammatory, antitumor and neuroprotective effects. However, the optimal dose required to exert a protective effect remains unclear.
(2) Previous studies have mainly used a focal cerebral ischemia model, this study established global cerebral ischemia/reperfusion injury in rats, in a broader attempt to optimize the protective dose of tea polyphenols on global cerebral ischemia/reperfusion injury in rats.
(3) We found that tea polyphenols can significantly reduce oxidative stress injury and apoptosis of nerve cells, and that the optimal protective dose is 200 mg/kg.

INTRODUCTION

Cerebral ischemia/reperfusion injury is a complex pathophysiological process, characterized by free radical generation, intracellular calcium overload, excitatory amino acid neurotoxicity, endoplasmic reticulum stress, and apoptosis. Overproduction of free radicals is the most critical event among these processes, and leads to oxidative stress injury. Neuronal death, caused by global cerebral ischemia/reperfusion injury, includes necrosis and apoptosis. Previous studies found that neuronal necrosis is caused by a cascade of reactions at early stages of cerebral ischemia/reperfusion injury, making it difficult to take protective measures effectively. Apoptosis, which is closely related to brain dysfunction caused by cerebral ischemia/reperfusion, is a type of delayed neuronal death, providing a good opportunity for clinical treatment[1]. Therefore, how to detect ischemia/reperfusion injury in time and take effective measures to block apoptosis is very important for the improvement of prognosis clinically.

Tea polyphenols are derivatives extracted from tea that are a class of chemicals containing polyphenol hydroxyls, and are famous for their strong anti-oxidative ability[2]. Although a few studies reported that tea polyphenols could alleviate cerebral ischemia/reperfusion injury[3], these studies were usually based on focal cerebral ischemia models and the effective dose was also inconsistent. In addition, little is known about the mechanisms underlying the protective, and anti-apoptotic role of tea polyphenols.

In this study, Sprague-Dawley rat models of global cerebral ischemia/reperfusion injury were treated with various doses of tea polyphenols immediately after reperfusion. Peroxide dismutase activity, malondialdehyde content and total antioxidant capacity were determined at six different time points to explore the optimal anti-oxidative dose of tea polyphenols. Furthermore, we observed neurobehavioral changes and neuronal apoptosis in the hippocampal CA1 region after intervention with an optimal dose of tea polyphenols.

Prior to manuscript drafting, our research group performed an online retrieval on paper innovation.
(1) Database retrieval: ISI Web of Knowledge Database from 2008 to 2011 was retrieved using the key words of cerebral ischemia and tea polyphenols. Ten relevant papers were screened out.
(2) Innovation of experimental theory: tea polyphenols could significantly alleviate oxidative stress injury and play crucial preventive roles on global cerebral ischemia/reperfusion injury.
(3) Innovation of experimental methods: global cerebral ischemia/reperfusion injury models were treated with various doses of tea polyphenols immediately after reperfusion. The activities of oxidative stress-related factors were determined to optimize the protective dose of tea polyphenols and to evaluate the influence of tea polyphenols on neuronal apoptosis in the hippocampal CA1 region and neurobehavioral outcomes in rats after global cerebral ischemia/reperfusion.
(4) Innovation of experimental outcomes: 200 mg/kg is the optimal dose of tea polyphenols for the prevention and treatment of global cerebral ischemia/reperfusion injury, and 200 mg/kg tea polyphenols play a crucial anti-apoptotic effect, reduce the rate of neuronal apoptosis in the hippocampal CA1 region, and improve neurobehavioral functions in rats.

RESULTS

Quantitative analysis of experimental animals

Five hundred rats were included in this study, 108 served as the sham surgery group (only the bilateral common carotid artery and vertebral artery were exposed, with no blocking and occlusion) and the remaining 392 were used to establish ischemia/reperfusion injury models. Twenty model rats were unable to tolerate the cerebral ischemia/reperfusion process and died, and another 12 model rats were excluded because of the absence of mydriasis 5 minutes after common carotid artery occlusion. In total, 468 rats were used in the final result analysis.

Among the 468 rats, 252 were randomly divided into seven groups: sham surgery group, model group, and tea polyphenol groups at 25, 50, 100, 150, 200 mg/kg. There were 36 rats per group, all for the detection of oxidative stress-related factors and optimization of the protective dose. In addition, 216 rats were randomly divided into a sham surgery group, model group, and 200 mg/kg tea polyphenol group, with 72 rats per group. Furthermore, each group was assigned into three subgroups, with 26 rats in each, for the terminal deoxynucleotidyl transferase-mediated nick end labeling assay, flow cytometry and neurobehavioral detection, respectively.

For rats in the sham surgery group, bilateral vertebral arteries were exposed but the carotid artery was not cauterized or occluded. In the tea polyphenol group, tea

polyphenols were intraperitoneally injected into rats immediately after reperfusion.

Effect of tea polyphenols on superoxide dismutase activity in brain tissue of rats after cerebral ischemia/reperfusion

To examine free radical generation during ischemia/reperfusion injury, we analyzed superoxide dismutase activity in the rat brain. Superoxide dismutase, a class of active substance derived from organisms, plays a vital role in diminishing noxious substances during the metabolic process. At the same time point, the model group showed the lowest superoxide dismutase activity compared with the sham surgery group ($P < 0.05$). The tea polyphenol group (200 mg/kg) showed the highest superoxide dismutase activity compared with the model group ($P < 0.05$). However, pairwise comparisons demonstrated that there was no significant difference between the different tea polyphenol groups ($P > 0.05$). Within the same dose group, superoxide dismutase activity decreased over time and reached the minimum levels at 48 hours (Figure 1).

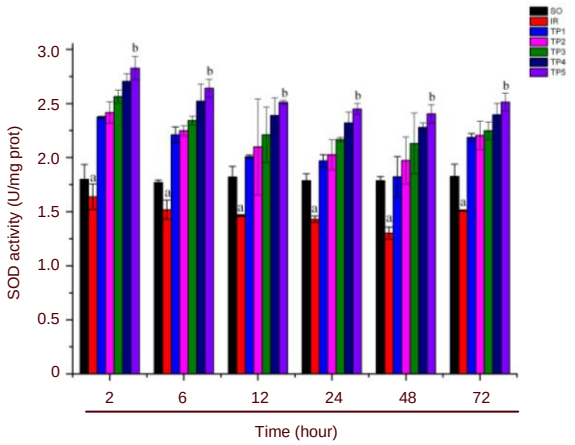

Figure 1 Effect of tea polyphenols on superoxide dismutase (SOD) in the brain tissue of rats with cerebral ischemia/reperfusion injury.

No significant difference was observed between different tea polyphenol groups ($P > 0.05$). Within the same treatment group, SOD activity decreased over time and reached control levels at 48 hours. [a]$P < 0.05$, *vs*. sham surgery group (SO); [b]$P < 0.05$, *vs*. model group (IR).

Data are expressed as mean ± SD, $n = 6$ in each group per time point. Statistical analysis was performed with multivariate analysis of variance and differences between groups were compared with the Student-Newman-Keuls test. TP1–5: Tea polyphenol 25, 50, 100, 150, 200 mg/kg groups.

Effect of tea polyphenols on malondialdehyde levels in the brain tissue of rats after cerebral ischemia/reperfusion

We further explored the protective role of tea polyphenols by examining malondialdehyde levels in the rat brain, which is a product of lipid peroxidation and a marker of oxidative stress. At the same time point, malondialdehyde levels were markedly increased in the model group compared with the sham surgery group ($P < 0.05$). The tea polyphenol group (200 mg/kg) achieved the largest reducing effect on malondialdehyde levels compared with the model group ($P < 0.05$). However, pairwise comparisons demonstrated that there was no significant difference between the different tea polyphenol groups ($P > 0.05$). Within the same dose group, malondialdehyde levels increased over time and peaked at 48 hours (Figure 2).

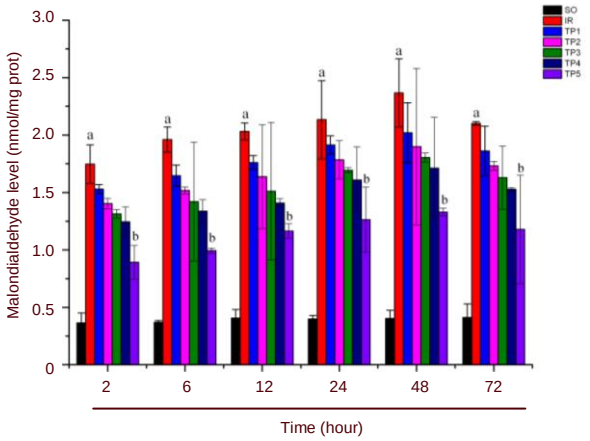

Figure 2 Effect of tea polyphenols on malondialdehyde levels in the brain tissue of rats after cerebral ischemia/reperfusion.

[a]$P < 0.05$, *vs*. sham surgery group (SO); [b]$P < 0.05$, *vs*. model group (IR). Data are expressed as the mean ± SD, $n = 6$ in each group. Statistical analysis was performed with multivariate analysis of variance, and differences between groups were compared with the Student-Newman-Keuls test. TP1–5: Tea polyphenol 25, 50, 100, 150, 200 mg/kg groups.

Effect of tea polyphenols on antioxidant levels in the brain tissue of rats after cerebral ischemia/reperfusion

We examined antioxidant capacity as an indicator of the protective role of tea polyphenols. At the same time point, total antioxidant capacity was markedly decreased in the model group compared with the sham surgery group ($P < 0.05$). The tea polyphenol group (200 mg/kg) resulted in the highest total antioxidant capacity activity ($P < 0.05$). However, pairwise comparisons demonstrated that there was no significant difference between the different tea polyphenol groups ($P > 0.05$). Within the same dose group, total antioxidant capacity decreased over time and reached the control levels at 48 hours (Figure 3).

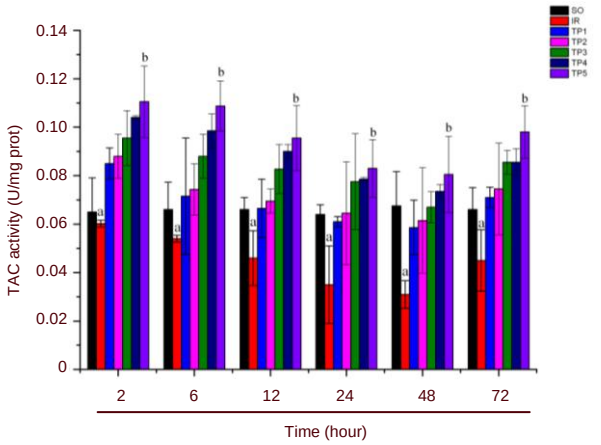

Figure 3 Effect of tea polyphenols (TP) on total antioxidant capacity (TAC) in the brain tissue of rats after cerebral ischemia/reperfusion.

At the same time point, TAC was significantly decreased after ischemia/reperfusion (IR) procedures, and this defect could be completely rescued by TP administration. No significant difference was observed between different TP doses ($P > 0.05$). Within the same treatment group, TAC decreased over time and reached basal levels at 48 hours. $^{a}P < 0.05$, *vs*. sham surgery group (SO); $^{b}P < 0.05$, *vs*. model group (IR).

Data are expressed as mean ± SD, $n = 6$ in each group. Statistical analysis was performed with multivariate analysis of variance, and differences between groups were compared with the Student-Newman-Keuls test. TP1–5: Tea polyphenol 25, 50, 100, 150, 200 mg/kg groups.

Effect of tea polyphenols on histological defects in the rat hippocampus induced by cerebral ischemia/reperfusion injury

Hematoxylin-eosin staining was applied to compare hippocampal histology under different treatments. In the sham surgery group, the hippocampal CA1 region showed clear structures with 3–4 layers at 2 and 48 hours after reperfusion. Pyramidal cells arranged closely in neat rows, with red-stained cytoplasm and big, round, and dark blue-stained nuclei. Approximately one or two trachychromatic nucleoli were observed in the center (Figures 4A, B). Under ischemia/reperfusion conditions, pyramidal cells in the CA1 region were disorganized, the number of neurons decreased, neuronal bodies shrank, the cytoplasm underwent eosinophilic degeneration, the nucleus became pyknotic, and the nucleoli disappeared (Figures 4C, D). Tea polyphenols (200 mg/kg) could partially rescue the histological defects induced by ischemia/reperfusion (Figures 4E, F).

Effect of tea polyphenols on neuronal apoptosis in the rat hippocampus induced by cerebral ischemia/reperfusion injury

The apoptotic levels in the hippocampus were detected with terminal deoxynucleotidyl transferase-mediated nick end labeling staining. Few apoptotic neurons were observed in the sham surgery group at 6 and 48 hours after reperfusion (Figures 5A, B). Ischemia/reperfusion markedly increased apoptotic levels, showing concentrated nuclei, bordered chromosomes, and typical apoptotic bodies (Figures 5C, D). The number of apoptotic neurons increased over time and peaked at 48 hours ($P < 0.05$; Figures 5C, D). Tea polyphenol administration (200 mg/kg) partially rescued the apoptosis induced by ischemia/reperfusion (Figures 5E, F). Apoptosis was further confirmed by Annexin V/FITC and propidium iodide double staining followed by flow cytometry analysis. Ischemia/reperfusion markedly increased apoptotic levels ($P < 0.05$), and this increase was partially reversed by 200 mg/kg tea polyphenol administration ($P < 0.05$; Figure 6).

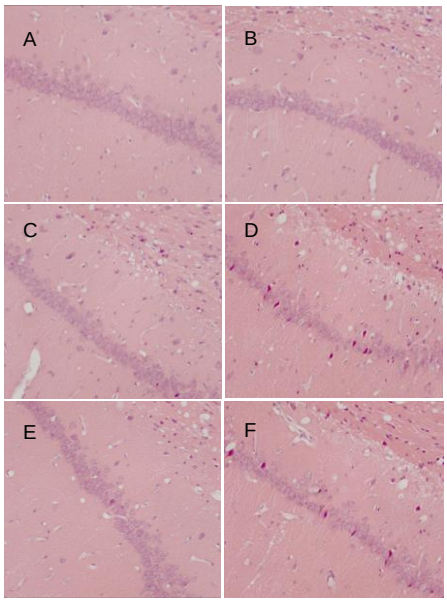

Figure 4 Effect of tea polyphenols on histological defects in the rat hippocampus induced by cerebral ischemia/reperfusion injury (light microscopy, hematoxylin-eosin staining, × 400).

As shown in the representative figures of the hippocampal CA1 region from the sham surgery group (A: 2 hours, B: 48 hours), the hippocampus showed clear structures with 3–4 layers. Pyramidal cells arranged closely in neat rows, with red-stained cytoplasm and big, round, and dark blue-stained nuclei. About one or two trachychromatic nucleoli were observed in the center.

Model group (C: 2 hours, D: 48 hours), pyramidal cells in the CA1 region were disorganized, the number of neurons was decreased, neuronal bodies shrank, the cytoplasm underwent eosinophilic degeneration, the nucleus became pyknotic, and nucleoli disappeared.

200 mg/kg tea polyphenol group (E: 2 hours, F: 48 hours) could partially rescue the histological defects induced by ischemia/reperfusion.

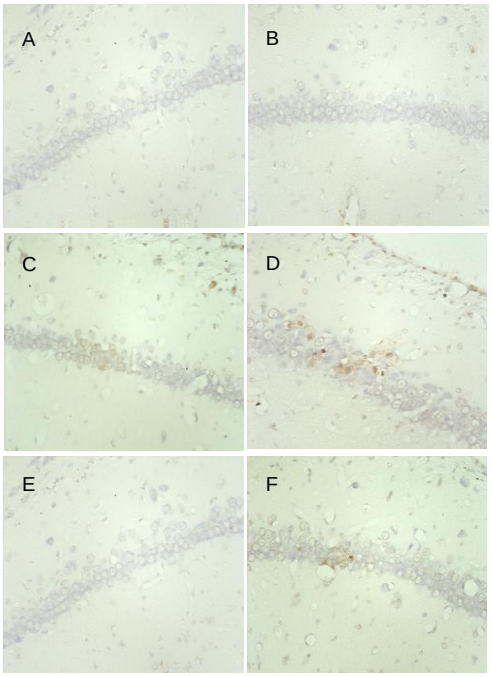

Figure 5 Effect of tea polyphenols on neuronal apoptosis in the rat hippocampus induced by cerebral ischemia/reperfusion injury (light microscopy, terminal deoxynucleotidyl transferase-mediated nick end labeling staining, × 400).

Shown are representative figures of hippocampal CA1 regions from the sham surgery group (A: 6 hours, B: 48 hours), model group (C: 6 hours, D: 48 hours), and 200 mg/kg tea polyphenol group (E: 6 hours, F: 48 hours).

At 6 and 48 hours after reperfusion, the number of apoptotic pyramidal cells significantly increased in the model group, showing concentrated nuclei, bordered chromosomes, and typical apoptotic bodies. Apoptosis levels peaked at 48 hours.

Effect of tea polyphenols on neurobehavioral outcomes of rats in the open field test following cerebral ischemia/reperfusion injury

To further examine the protective role of tea polyphenols after ischemia/reperfusion, we used the open field test, which measures behavioral responses of rats, to reflect anxiety levels. At 24, 48, 72 hours after ischemia/reperfusion, both the time spent in the center (Table 1) and the distance travelled (Table 2) in the open field were significantly reduced following ischemia/reperfusion, and this reduction was partially rescued by 200 mg/kg tea polyphenol administration ($P < 0.05$). The two indices in the tea polyphenol group were significantly decreased compared with the sham surgery group and increased compared with the model group ($P < 0.05$; Tables 1, 2).

Effect of tea polyphenols on neurobehavioral outcomes using the elevated plus maze test following cerebral ischemia/reperfusion injury

We further examined the anxiety level of rats using the elevated plus maze test. At 24, 48 and 72 hours after reperfusion, both the percentage of open arm entries (Table 3) and time spent in the open arms (Table 4) were significantly reduced following ischemia/reperfusion ($P < 0.05$), and this reduction was partially rescued by 200 mg/kg tea polyphenol administration ($P < 0.05$). There were significant differences between the tea polyphenols and the model groups, and tea polyphenols and sham surgery groups ($P < 0.05$).

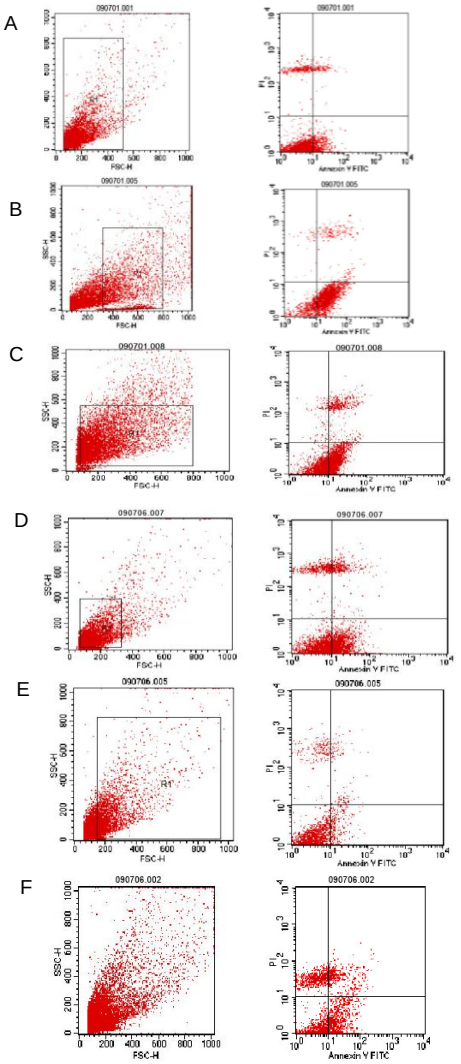

Figure 6 Effect of tea polyphenols (TP) on neuronal apoptosis in the rat hippocampus induced by cerebral ischemia/reperfusion injury (Annexin V/Fluorescein isothiocyanate and propidium iodide double staining, flow cytometry).

Shown are representative flow cytometry diagrams from the sham surgery group (A: 6 hours; D: 48 hours), model group (B: 6 hours; E: 48 hours), and TP group (C: 6 hours; F: 48 hours), $n = 6$ in each group. In the two-parametric scatter diagrams, the left lower quadrant represented normal cells, right lower quadrant apoptotic cells in their early stage, and left upper quadrant necrotic cells. The number of apoptotic cells in the hippocampal CA1 region was lower in the TP group than in the model group.

Table 1 Percentage of time spent in the center (%) by rats after global ischemia/reperfusion in the open field test

Group	Time after ischemia/reperfusion (hour)		
	24	48	72
Sham surgery	6.44±0.78	6.40±0.88	6.45±0.86
Model	3.61±0.48	2.34±0.50	1.45±0.31
Tea polyphenol (200 mg/kg)	4.67±0.54[ab]	3.78±0.73[ab]	2.50±0.72[ab]

[a]$P < 0.05$, *vs*. sham surgery group; [b]$P < 0.05$, *vs*. model group. Data are expressed as mean ± SD, $n = 6$ in each group. Statistical analysis was performed with multivariate analysis of variance, and differences between groups were compared with the Student-Newman-Keuls test. The time spent in the central activity = time spent in the dark center activity/time spent in the dark to the total field activity (%).

Table 2 Percentage of distance travelled (%) by rats after global ischemia/reperfusion in the open field test

Group	Time after ischemiare/perfusion (hour)		
	24	48	72
Sham surgery	6.76±0.74	6.33±0.73	7.00±0.71
Model	3.60±0.64	2.93±0.66	1.93±0.64
Tea polyphenol (200 mg/kg)	5.56±0.62[ab]	4.68±0.73[ab]	3.30±0.72[ab]

[a]$P < 0.05$, *vs*. sham surgery group; [b]$P < 0.05$, *vs*. model group. Data are expressed as the mean ± SD, $n = 6$ in each group. Statistical analysis was performed with multivariate analysis of variance, and differences between groups were compared with the Student-Newman-Keuls test.

Table 3 Percentage of open arm entries (%) by rats after global ischemia/reperfusion in the elevated plus maze test

Group	Time after ischemia/reperfusion (hour)		
	24	48	72
Sham surgery	42.20±3.10	43.40±3.48	42.77±2.85
Model	28.24±1.95	23.53±1.83	19.07±2.04
Tea polyphenol (200 mg/kg)	32.72±2.49[ab]	29.67±2.84[ab]	23.25±2.34[ab]

[a]$P < 0.05$, *vs*. sham surgery group; [b]$P < 0.05$, *vs*. model group. Data are expressed as mean ± SD, $n = 6$ in each group. Statistical analysis was performed with multivariate analysis of variance, and differences between groups were compared with Student-Newman-Keuls test.

DISCUSSION

Overproduction of free radicals is an important mechanism underlying cerebral ischemia/reperfusion injury, causing oxidative stress, excitotoxicity, ionic imbalance, and apoptosis or necrosis of brain cells[4]. Tea polyphenols and their derivatives, with epigallocatechin gallate as the main active ingredient, exert anti-oxidative, anti-inflammatory, and anti-tumor effects. Growing evidence has reported the protective role of tea polyphenols in rats and mice with cerebral ischemia/reperfusion injury[5-7]. A previous study compared tea polyphenol administration at 0 or 30 minutes after reperfusion, and found that the protective effect of tea polyphenols was more significant when injected immediately after reperfusion[8]. Thus, immediate injection of tea polyphenols was employed in this study.

Table 4 Percentage of time spent in the open arm (%) by rats after global ischemia/reperfusion in the elevated plus maze test

Group	Time after ischemia/reperfusion (hour)		
	24	48	72
Sham surgery	42.61±3.33	42.06±4.31	42.01±3.14
Model	25.73±2.87	21.69±2.37	18.34±2.73
Tea polyphenol (200 mg/kg)	32.82±3.44[ab]	28.37±2.49[ab]	24.45±2.77[ab]

[a]$P < 0.05$, *vs*. sham surgery group; [b]$P < 0.05$, *vs*. model group. Data are expressed as mean ± SD, $n = 6$ in each group. Statistical analysis was performed with multivariate analysis of variance, and differences between groups were compared with the Student-Newman-Keuls test.

To our knowledge, the protective dose of tea polyphenols has not been optimized and doses used in previous studies ranged from 25 mg/kg to several hundred mg/kg of body weight. In addition, previous studies addressing tea polyphenols used incomplete cerebral ischemia/reperfusion injury models in rats[1], and little is known about the effects of tea polyphenols in global cerebral ischemia/reperfusion injury. In this study, we compared the effects of different doses of tea polyphenols (25, 50, 100, 150, and 200 mg/kg) on global cerebral ischemia/reperfusion injury. Our results showed that global cerebral ischemia/reperfusion significantly increased malondialdehyde levels and decreased superoxide dismutase and total antioxidant capacity, confirming that oxidative stress is an important mechanism leading to cerebral ischemia/reperfusion injury. This oxidative stress was most intense at 48 hours. Tea polyphenol administration partially (for malondialdehyde levels) or completely (for superoxide dismutase and total antioxidant capacity) reduced the oxidative stress induced by ischemia/reperfusion injury. Tea polyphenol administration (200 mg/kg) showed the strongest anti-oxidative intervention and this dosage was used in all following studies. However, pairwise comparisons demonstrated that there was no significant difference between different dosage groups. We next showed that tea polyphenols protected hippocampal cells from histological defects (hematoxylin-eosin staining) and apoptosis (Terminal deoxynucleotidyl

transferase-mediated nick end labeling staining, and Annexin V/Fluorescein isothiocyanate and propidium iodide double staining) induced by ischemia/reperfusion injury. Under ischemia/reperfusion injury, the apoptotic levels in the hippocampus increased significantly from 6 hours, peaked at 48 hours, and declined afterwards. This observation suggested that apoptosis occurs relatively late during cerebral ischemia/reperfusion injury, indicating a good treatment opportunity. Different from previous studies[9], which showed that the cortex has more collateral circulation and is more tolerant to ischemia than the hippocampus, we observed that 6–48 hours after cerebral ischemia/reperfusion, cortical neurons showed more pronounced apoptosis than hippocampal neurons (data are not shown). Future studies are required to examine the mechanisms underlying the protective role of tea polyphenols as well as the effects of ischemia/reperfusion on cortical neurons. Because the hippocampus plays an important role in regulating anxiety, we further examined whether tea polyphenols can decrease anxiety levels following cerebral ischemia/reperfusion injury. Tea polyphenols significantly improved the exploratory behavior of native Wister rats in the open-field test[10], water maze test, and passive avoidance test. In our study, tea polyphenols significantly improved neurobehavioral outcomes of both the open field test and elevated plus maze test, suggesting that tea polyphenols can improve exploratory behavior and suppress anxiety in rats under cerebral ischemia/reperfusion injury.

This experiment used higher doses of tea polyphenols in the pre-experimental phase, but found that more than 200 mg/kg led to a very high animal mortality rate, so the present study did not explore the higher dose. In conclusion, we found that the optimum protective dose of tea polyphenols was 200 mg/kg. When administered immediately after ischemia/reperfusion, tea polyphenols can reduce oxidative stress, rescue histological defects, and decrease apoptosis in the hippocampus of rats. Tea polyphenol administration also improves exploratory behavior and reduces the anxiety levels of rats following ischemia/reperfusion injury.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

Experiments were completed from July 2008 to December 2008 in Animal Laboratory of Xi'an Jiaotong University, China.

Materials

Five hundred healthy and clean male Sprague-Dawley rats, aged 55–65 days, weighing 300 ± 20 g, were provided by the Experimental Animal Cultivation Center of Xi'an Jiaotong University in China. Animals were fed in the specific pathogen free-level laboratory for 1 week, and were confirmed to have no abnormal behavior before examination. All rats were fasted overnight, but allowed to drink water freely before surgery. All the procedures were in compliance with the *Guide for the Care and Use of Laboratory Animals* issued by the National Institute of Health (NIH Publications #85-23, revised in 1985).

Methods

Establishment of global cerebral ischemia/reperfusion models

Global cerebral ischemia/reperfusion was established using the four-vessel occlusion method[11]. The rats received intraperitoneal injection of 10% (v/v) chloralhydrate. After rats were fixed in the supine position, a median incision was made at the level of the first cervical vertebrae under the occipital bone. The muscle was then separated layer by layer to expose the small wing hole in the transverse process of the first cervical vertebrae. A hot probe was inserted into the small wing hole to cauterize and thus permanently occlude the bilateral vertebral arteries. The postoperative rats were housed separately in cages at 20–25°C. Meanwhile, a 60-W incandescent lamp was kept on for 24 hours. The second day after operation, the rats were again injected with chloralhydrate and fixed in the supine position as described. After a median incision was made at the neck, bilateral carotid arteries were separated and obturated with bulldog clamps. One minute later, the rats showed mydriasis, pale eyeballs, lip cyanosis, and ecphysesis. Five minutes later, the bulldog clamps were released and reperfusion began. Any animal that did not accord with this standard or appeared hemiplegic was not included. For rats in the sham surgery group, bilateral vertebral arteries were exposed but the carotid artery was not cauterized or occluded. Rats were sacrificed as previously described.

Oxidative stress analysis

The rats in the sham surgery, model, and tea polyphenol groups received intraperitoneal injection of 2 mL saline, saline, and tea polyphenols (Sigma, St. Louis, MO, USA; CAS: 84650-60-2) of corresponding concentrations,

respectively. Rats were sacrificed at the indicated time points and brain tissue was made into 10% (w/v) homogenates with cold saline. Protein content of samples was determined using the Folin-phenol kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China). The levels of superoxide dismutase, malondialdehyde, and total antioxidant capacity in brain tissues were determined using the corresponding kits (Nanjing Jiancheng Bioengineering Institute).

Terminal deoxynucleotidyl transferase-mediated nick end labeling detection for hippocampal neuronal apoptosis

Hippocampal coronal paraffin sections were dewaxed in xylene and ethanol, and processed with the terminal deoxynucleotidyl transferase-mediated nick end labeling apoptosis kit (Roche Co., Indianapolis, IN, USA). Neurons in the hippocampal CA1 region were observed under a light microscope, and apparent brown particles or patches were regarded as positive cells. Slides were immersed in 10% (v/v) buffered formalin or 4% (w/v) paraformaldehyde for 25 minutes and in 0.2% (v/v) Triton X-100 in PBS for 5 minutes, then rinsed twice with PBS, and equilibrated with 100 µL equilibration buffer at room temperature for 5–10 minutes. Cells on the slides were mixed with 100 µL of TdT reaction buffer, so that they would not dry completely. Slides were covered with plastic coverslips to ensure even distribution of the mixture. Slides were incubated for 60 minutes at 37°C in a humidified chamber. After plastic coverslips were removed, slides were immersed in 2 × saline-sodium citrate for 15 minutes and in 0.3% (v/v) hydrogen peroxide for 3–5 minutes. Then slides were incubated with 100 µL streptavidin-horseradish peroxidase (diluted 1:500 in PBS) for 30 minutes at room temperature. Slides were developed with 100 µL 3,3'-diaminobenzidine immediately prior to use, until a light brown background appeared. The background was forbidden to become too dark. Slides were rinsed with deionized water and mounted in an aqueous or permanent mounting medium, and finally observed under a light microscope (Olympus, Tokyo, Japan).

Flow cytometry detection for neuronal apoptosis

Six rats were sacrificed at the indicated time points following reperfusion. The whole brain was dissected and kept in an ice tray containing cold PBS. After the pia mater and arachnoid were peeled off, tissue between the optic chiasm and mammillary body, about 0.5 cm × 0.5 cm, was kept cold and resuspended into a single cell suspension at 1×10^6 cells/mL within 30 minutes. Cells were double stained with Annexin V/Fluorescein isothiocyanate and propidium iodide (Jingmei BioTech Co., Ltd., Xi'an city, Shaanxi Province, China) for 15 minutes in the dark and then analyzed by flow cytometry (FACSCalibur, BD Becton Dickinson, San Jose, CA, USA).

Open field test for rat neurobehavioral changes

Rat neurobehavioral changes were detected using an open field box (Yishu Technology Co., Ltd., China), 40 cm high and 100 cm long, and its walls and bottom were colored black. A digital camera, 2 m above the box, was used to record the activity of rats. Experiments were conducted in a quiet environment with minimal interference. Rats were placed in the middle of an open field box and their activities were recorded for 5 minutes. Main outcome measures were the percentage of time spent in the center and distance travelled in the center. After each test, the walls and bottom of the box were cleaned with 10% (v/v) alcohol to avoid the influence of rat urination and defecation on test results.

Elevated plus maze test for rat neurobehavioral changes

The elevated plus maze (Yishu Technology Co., Ltd., China) consisted of two open arms (50 cm × 10 cm), two closed arms (50 cm × 10 cm × 40 cm), and a central platform (10 cm × 10 cm), which connected the four arms. During experiments, the laboratory environment was kept quiet, the lighting was subdued so that only objects within 1.5 m could be distinguished, and the temperature was maintained at 25°C. The rat was placed in a separate plastic box for 5 minutes before the test, and then quickly placed on the central platform with its head towards an open arm. The recording started simultaneously and lasted 5 minutes. Activity started when four paws of a rat entered an arm, and ended when any one paw retreated from the arm. The percentages of time spent in the open arm and the number of open arm entries were recorded. After each test, the arms and the central platform were cleaned with 10% (v/v) alcohol.

Statistical analysis

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Multivariate analysis of variance was adopted to compare the relationship between the outcome variable and the dose. If a relationship was found, the Student-Newman-Keuls test was used to make pairwise comparisons to select the maximally protective dose. A $P < 0.05$ value was considered statistically significant.

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