Tea polyphenols increase X-ray repair cross-complementing protein 1 and apurinic/apyrimidinic endonuclease/redox factor-1 expression in the hippocampus of rats during cerebral ischemia/reperfusion injury*  

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Abstract
Recent studies have shown that tea polyphenols can cross the blood-brain barrier, inhibit apoptosis and play a neuroprotective role against cerebral ischemia. Furthermore, tea polyphenols can decrease DNA damage caused by free radicals. We hypothesized that tea polyphenols repair DNA damage and inhibit neuronal apoptosis during global cerebral ischemia/reperfusion. To test this hypothesis, we employed a rat model of global cerebral ischemia/reperfusion. We demonstrated that intraperitoneal injection of tea polyphenols immediately after reperfusion significantly reduced apoptosis in the hippocampal CA1 region; this effect started 6 hours following reperfusion. Immunohistochemical staining showed that tea polyphenols could reverse the ischemia/reperfusion-induced reduction in the expression of DNA repair proteins, X-ray repair cross-complementing protein 1 and apurinic/apyrimidinic endonuclease/redox factor-1 starting at 2 hours. Both effects lasted at least 72 hours. These experimental findings suggest that tea polyphenols promote DNA damage repair and protect against apoptosis in the brain.

Key Words
global cerebral ischemia/reperfusion; X-ray repair cross-complementing protein 1; apurinic/apyrimidinic endonuclease/redox factor-1; tea polyphenols

Research Highlights
(1) During the early period after global cerebral ischemia/reperfusion, tea polyphenols play an important neuroprotective role by reversing the reduction in the expression of DNA repair proteins X-ray repair cross-complementing protein 1 and apurinic/apyrimidinic endonuclease/redox factor-1.
(2) Tea polyphenols promote DNA damage repair.
(3) Tea polyphenols protect against apoptosis in hippocampal cells.

Abbreviations
XRCC1, X-ray repair cross-complementing protein 1; APE/Ref-1, apurinic/apyrimidinic endonuclease/redox factor-1
INTRODUCTION

Numerous studies have focused on the mechanisms associated with cerebral ischemia/reperfusion injury, including DNA damage/repair and apoptosis[1]. Apoptosis is a highly-regulated, time-dependent progress during cerebral ischemia/reperfusion. The extent of apoptosis increases as reperfusion continues and DNA damage accumulates[2-3]. Furthermore, studies have shown that ischemia impairs DNA repair functions[4-5]. The DNA repair system prevents cell death by repairing damaged DNA, but overwhelming impairment of DNA repair can result in apoptosis[6]. DNA repair, such as base excision repair of DNA single-strand breaks, plays a key role in the maintenance of genomic integrity and the regulation of cell cycle checkpoints following DNA damage[7-8].

The DNA repair protein, X-ray repair cross-complementing protein 1 (XRCC1), plays a critical role in DNA single strand break repair. As an important participant in the base excision repair pathway, XRCC1 up-regulates the activity of DNA ligase III, apurinic/apyrimidinic endonuclease and polynucleotide kinase, and inhibits poly(ADP-ribose) polymerase-1/2 and DNA polymerase β[9]. XRCC1-deficient cells show an increase in cell death and a decrease in single-stranded DNA repair[10]. Previous studies have shown that during brain injury, a reduction in XRCC1 levels at the early stage is an important factor contributing to DNA damage and apoptosis[11].

Apurinic/apyrimidinic endonuclease/redox factor-1 (APE/Ref-1) is a multifunctional enzyme in the base excision repair pathway responsible for repairing apurinic/apyrimidinic sites in DNA, which are generated by oxidative stress after ischemia/reperfusion[12]. During the early stage of cerebral ischemia/reperfusion, free radicals can cause several types of DNA damage, including DNA apurinic/apyrimidinic sites, base damage and single-stranded and double-stranded DNA breaks[13-14]. Vidal et al[15] showed that XRCC1 contributes to apurinic/apyrimidinic site repair by interacting with and stimulating the activity of APE/Ref-1. APE/Ref-1 can recognize and repair apurinic/apyrimidinic sites and base damage, function as a 3’-phosphodiesterase and repair single-stranded and double-stranded DNA breaks[16]. This DNA damage mediated by reactive oxygen species is an important mechanism underlying neuronal DNA damage. APE/Ref-1 is the rate-limiting enzyme in the defense against reactive oxygen species damage[17-18]. Furthermore, APE/Ref-1 also participates in redox regulation by regulating the DNA binding activities of various transcription factors, including activator protein-1 and nuclear factor κB[19]. These transcription factors further contribute to neuronal DNA damage repair and defend the brain against apoptosis.

Tea polyphenols, first isolated from green tea, have a structure similar to glutamate. Recent studies have shown that tea polyphenols can penetrate the blood-brain barrier, inhibit the release of excitatory amino acids, improve microcirculation, reduce apoptosis, interact with inflammatory factors and neurotransmitters, and protect the brain against cerebral ischemia[20-21]. Recent studies have shown that tea polyphenols can effectively suppress spiral ganglion neuron apoptosis by down-regulating the expression of Bax, caspase-3 and caspase-6, and up-regulating the expression of Bcl-2[22-23]. Tea polyphenols can also reduce DNA damage induced by free radicals[23-25]. This effect has been attributed to their potent antioxidant and free radical scavenging actions[26]. However, little is known about how tea polyphenols affect DNA damage and neuronal apoptosis during cerebral ischemia/reperfusion.

In this study, we employed a rat model of global cerebral ischemia/reperfusion injury to examine the expression of the DNA repair proteins XRCC1 and APE/Ref-1 in the hippocampal CA1 region during ischemia/reperfusion injury. We then explored the protective role of tea polyphenols in this process. This study contributes to the current understanding of the mechanisms underlying cerebral ischemia/reperfusion injury, and provides insight into how brain damage can be alleviated.

RESULTS

Quantitative analysis of experimental animals

A total of 108 rats were initially included in the study and were randomly divided into three groups: a sham group (SH), an ischemia/reperfusion group (IR) and an ischemia/reperfusion followed by tea polyphenols treatment group (TP). Rats were sacrificed 2, 6, 12, 24, 48 or 72 hours after reperfusion. There were 6 rats per group per time point. All 108 rats were included in the final analysis.

Tea polyphenols reduce apoptosis in the hippocampus induced by cerebral ischemia/reperfusion injury

We examined apoptosis in the hippocampus using TUNEL staining, and subsequently calculated the apoptosis index (Figure 1). In this experiment, the apoptotic index was used to assess the level of apoptosis. Ischemia/reperfusion significantly increased apoptosis at 6 hours, and the apoptotic level peaked at 48 hours (P <
Tea polyphenols suppress the reduction in XRCC1 expression in the hippocampus induced by cerebral ischemia/reperfusion injury

We next examined the expression of DNA repair protein XRCC1 following ischemia/reperfusion by immunohistochemistry (Figure 2). Under ischemia/reperfusion injury, the percentage of XRCC1-positive cells decreased significantly from 2–72 hours ($P < 0.01$, vs. SH group). This reduction could be suppressed by administration of tea polyphenols ($P < 0.01$, vs. IR group).

DISCUSSION

Fujimura et al. [27] reported that an early reduction of XRCC1 expression preceded DNA fragmentation in the region supplied by the (occluded) middle cerebral artery,
and that this reduction may contribute to DNA damage-induced cell death after transient focal cerebral ischemia in mice.

Figure 3  Tea polyphenols suppress the reduction in apurinic/apyrimidinic endonuclease/redox factor-1 (APE/Ref-1) expression induced by cerebral ischemia/reperfusion injury (immunohistochemistry staining, × 400).

Rats were treated as in Figure 1. Shown are representative APE/Ref-1 immunohistochemistry figures from the hippocampal CA1 region at 48 hours for the SH group (A), IR group (B) and TP group (C). Arrows show APE/Ref-1-positive cells.

D shows the percentage of APE/Ref-1-positive cells from the three groups at different time points. Under ischemia/reperfusion injury, the percentage of APE/Ref-1-positive cells decreased significantly from 2-72 hours \( (P < 0.01, \text{ vs. SH group}) \). Tea polyphenols suppressed the reduction in APE/Ref-1 expression induced by ischemia/reperfusion injury \( (P < 0.01, \text{ vs. IR group}) \).

Data are presented as mean ± SD, \( n = 6 \) per group per time point. Different groups were compared using analysis of variance for two-factor factorial design and least-significant difference t-test.

SH: Sham treated group; IR: ischemia/reperfusion group; TP: tea polyphenols group.

Our present results indicate that expression of XRCC1 and APE/Ref-1 start to decrease 2 hours after global cerebral ischemia/reperfusion, and remain low for up to 72 hours. Moreover, apoptosis is increased at 6 hours after ischemia/reperfusion, reaching a peak at 48 hours, and showing a subsequent gradual reduction. Combined with results from flow cytometry, we determined that expression of both XRCC1 and APE/Ref-1 negatively correlated with apoptotic rate. Furthermore, the decrease in XRCC1 and APE/Ref-1 expression occurred prior to apoptosis. These findings provide further evidence that the early reduction of XRCC1 after global cerebral ischemia/reperfusion is associated with the subsequent occurrence of DNA fragmentation. DNA fragmentation is possibly due to a large number of DNA single-strand breaks. The early reduction in XRCC1 protein levels result in reduced DNA single-strand break repair capacity. Consequently, a large number of DNA single-strand breaks accumulate in neurons, leading to DNA fragmentation in the late stage of ischemia/reperfusion, which ultimately results in apoptosis.

Kawase et al\(^2\) demonstrated that APE/Ref-1 decreases after transient global ischemia, and that this reduction precedes apoptosis. Moreover, APE/Ref-1 immunohistochemistry combined with TUNEL staining revealed that all TUNEL-positive CA1 pyramidal neurons were devoid of APE/Ref-1 immunoreactivity. In addition, quantitative analysis revealed that the decrease in the number of APE/Ref-1-immunopositive cells preceded the increase in the number of TUNEL-positive cells. We obtained similar results, suggesting that the loss of APE/Ref-1 and the failure of DNA repair might contribute to neuronal apoptosis in the hippocampal CA1 region after global cerebral ischemia.

The exact mechanism by which the reduction of XRCC1 and APE/Ref-1 occurs after ischemia/reperfusion is unclear. A study using the transient focal cerebral ischemia model demonstrated that mice overexpressing the endogenous antioxidant enzyme superoxide dismutase-1 show less reduction of APE/Ref-1 after ischemia/reperfusion compared with wild-type mice\(^3\). It is well known that reperfusion increases mitochondrial production of superoxide radicals\(^\#\). Therefore, it is conceivable that the mechanism underlying the reduced expression of XRCC1 and APE/Ref-1 is linked to oxidative stress. Further studies of DNA repair using mutant mice deficient in and/or overexpressing SOD are required to clarify this important issue.

Recently, green tea has been reported to have antioxidant properties\(^3\), and many researchers have investigated the cerebral protective activity of tea polyphenols. Previous studies have shown that tea polyphenols protect against DNA damage and reduce neuronal apoptosis after transient cerebral ischemia/reperfusion in gerbils\(^2\). In our experiment, we employed a rat model of global cerebral ischemia/reperfusion and demonstrated that intraperitoneal injection of tea polyphenols immediately after reperfusion significantly reduces apoptosis in the hippocampal CA1 region. Tea polyphenols suppressed the ischemia/reperfusion-induced reduction in the expression of DNA repair proteins XRCC1 and Ref-1. At 2 hours after cerebral ischemia/reperfusion and intraperitoneal injection of tea polyphenols, XRCC1 and APE/Ref-1 expression were significantly increased in the TP group compared with the IR group, and remained significantly elevated up to
These trends are consistent with the changes in the apoptotic levels revealed by TUNEL staining, suggesting that tea polyphenol-induced up-regulation of XRCC1 and Ref-1 may play an important role in reducing apoptosis during ischemia/reperfusion. The mechanisms underlying the neuroprotective effect of tea polyphenols are not fully understood. Nonetheless, it seems that the potent antioxidant effects of tea polyphenols contribute to its neuroprotective effect against neuronal damage after cerebral ischemia/reperfusion. We propose that oxidative stress decreases XRCC1 and APE/Ref-1 expression in ischemia/reperfusion, and that tea polyphenols exert their antioxidant effects by increasing expression of these critical proteins, which leads to reduced apoptosis in the hippocampal CA1. However, further experiments are required to clarify this concept. In conclusion, during global cerebral ischemia/reperfusion, tea polyphenols play an important neuroprotective role by suppressing the reduction in the expression of the DNA repair proteins XRCC1 and APE/Ref-1. This promotes DNA damage repair and protects against apoptosis in the brain. Future studies are required to examine how tea polyphenols affect the expression of XRCC1 and APE/Ref-1.

MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
The study was performed at Medical College of Xi’an Jiaotong University, China, from March 2009 to January 2010.

Materials

Animals
All the study procedures were in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). Healthy male Sprague-Dawley rats, aged 55–65 days and weighing 290–310 g, were provided by the Experimental Animal Center of Xi’an Jiaotong University School of Medicine, China (certificate No. SCXK (Shaan) 2007-001). All rats were fasted overnight and had free access to water the night before the operation.

Reagents
Tea polyphenols (powder) were purchased from Shanghai Yuanye Biological Technology Co., Ltd., Shanghai, China; CAS 84650-60-2. Tea polyphenols (25 g) and double distilled water (500 mL) were added into a beaker on a magnetic stirrer and stirred until the tea polyphenols were completely dissolved. This 5% tea polyphenols solution was transferred to a bottle, sealed under sterile conditions, and stored at 4°C.

Methods

Rat model of global cerebral ischemia/reperfusion and drug administration
A Sprague-Dawley rat model of global cerebral ischemia/reperfusion was established using the four-vessel occlusion method as previously described. Briefly, for rats from the IR and TP groups, the carotid artery was occluded using a micro arterial clamp. 5 minutes later, the clamp was loosened to restore cerebral artery blood flow. For rats in the SH group, the bilateral carotid arteries and vertebral arteries were exposed, but the bilateral carotid arteries were not occluded and the vertebral arteries were not cauterized. Immediately after cerebral ischemia/reperfusion, rats received intraperitoneal injection of 4 mL/kg of saline (for the SH and IR groups) or 4 mL/kg (200 mg/kg) of 5% tea polyphenols solution (for the TP group).

Brain tissue preparation
Rats were sacrificed at the indicated time points with heart perfusion of 200 mL of 4% paraformaldehyde. The brain regions that contained the hippocampal CA1 region (1–4 mm posterior to the chiasma opticum) were quickly removed and further fixed for 12 hours in 4% paraformaldehyde at 4°C. Post-fixed brains were embedded in paraffin, followed by preparation of 5-μm thick coronal sections using a microtome.

TUNEL staining
The hippocampal paraffin blocks were dewaxed in xylene and ethanol, digested with Proteinase K (20 mg/L) at room temperature, treated with hydrogen peroxide in methanol, incubated first with terminal deoxynucleotidyl transferase and biotinylated nucleotide (biotin-11-dUTP), then with streptavidin-biotin horseradish peroxidase solution, and finally developed with diaminobenzidine. Cells with dark brown nuclei were apoptotic cells. The hippocampal CA1 region was observed under a light microscope (× 400; B-type biomicroscope; Global Motic Group, Xiamen, China). Five fields were randomly selected to calculate the apoptosis index, defined as the number of apoptotic cells/the total number of cells \( \times 100\% \). TUNEL apoptosis detection kit was purchased from Promega Corp., WI, USA. Streptavidin biotin complex (rabbit IgG)-peroxidase kit was from Boster.
Biotechnology, Wuhan, Hubei Province, China. Diaminobenzidine substrate kit was purchased from Beijing Biosynthesis Biotechnology Co., Ltd., China.

**Immunohistochemistry**

The hippocampal paraffin blocks were sectioned and processed for immunohistochemistry using the avidin-biotin-peroxidase method[38]. Briefly, sections were dewaxed, treated with hydrogen peroxide in methanol for 10 minutes, washed with triple distilled water for 5 minutes, incubated in PBS (pH 7.4) for 5 minutes, and heated to 95°C for 15-20 minutes. After being cooled down to room temperature, the sections were first blocked with normal goat serum at room temperature for 1 hour, and then incubated with rabbit anti-XRCC1 polyclonal antibody (1:100; Beijing Biosynthesis Biotechnology Co., Ltd.) or rabbit anti-Ref-1 polyclonal antibody (1:150) at 4°C overnight. After three 5-minute washes in PBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody (1:100) at 37°C for 1 hour. After three 5-minute washes in PBS, sections were incubated with streptavidin biotin complex at 37°C for 20 minutes, washed three times with triple distilled water for 5 minutes each, and developed with diaminobenzidine. Cells with brown granules in the cytoplasm were XRCC1- or APE/Ref-1-positive cells. The hippocampal CA1 region was observed under a light microscope (× 400). Five fields were randomly selected to calculate the percentage of positive cells, defined as the number of positive cells/the total number of cells × 100%.

**Statistical analysis**

Data were analyzed using SPSS13.0 software (SPSS, Chicago, IL, USA). Different groups were compared using analysis of variance for two-factor factorial design and least-significant difference t-test. \( P < 0.05 \) was considered statistically significant.

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**Author contributions:** Rongliang Xue was responsible for funding, designed this study and revised the manuscript. Zhi Wang provided experimental data, participated in statistical analysis and wrote the manuscript. Xi Lei and Jianrui Lv revised the first version of the manuscript. Gang Wu and Wei Li provided technical support and performed statistical calculations. Li Xue and Xiaoming Lei provided information support. Hongxia Zhao, Hui Gao and Xin Wei participated in experimental animal breeding and processing.

**Conflicts of interest:** None declared.

**Ethical approval:** This study was approved by the Medical Ethics Committee of Xi’an Jiaotong University, China.

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