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PROTECTIVE EFFECT OF TEA POLYPHENOLS ON THE BLOOD-BRAIN BARRIER

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Abstract

This study was to investigate the protective effects of tea polyphenols on the blood-brain barrier (BBB) of rats with global cerebral ischemia/reperfusion (GCIR) injury. Sprague Dawley rats underwent four-vessel occlusion to construct the model of GCIR. Half an hour before complete occlusion, they were treated with tea polyphenols (TP) (6.4%; 100 or 200 mg/kg) via tail intravenous injection. 24 h after reperfusion, BBB permeability was evaluated by measuring brain water content (BWC) and residual amount of Evan's blue dye in cerebral tissue. In addition to this, MMP-9 and collagen IV protein expression in cerebral tissue were also detected using immunohistochemistry. ANOVA and SNK-q were used to do statistical analysis. Statistical significance was considered at $P<0.05$. Compared to the untreated, the TP-treated rats had significantly decreased BWC ($P<0.05$), decreased residual amount of Evan's blue dye in cerebral tissue ($P<0.05$), down-regulated MMP-9 ($P<0.05$) and up-regulated collagen IV expression in brain tissue ($P<0.05$). It can be concluded from these findings that TP may reduce the MMP-9 mediated collagen IV degradation caused by GCIR to protect the BBB.

Keywords

• Global cerebral ischemia/reperfusion (GCIR) • Blood–brain barrier (BBB) • Tea polyphenols (TP) • Collagen IV • Matrix metallopeptidase 9 (MMP-9)

Introduction

Global cerebral ischemia/reperfusion (GCIR) injury occurs commonly in patients with cardiac arrest, shock and asphyxia and in those patients undergoing complex cardiac surgery [1]. Studies demonstrate that GCIR may undermine the integrity of the blood-brain barrier (BBB) [2]. The BBB is formed by the endothelial cells of cerebral microvessels and serves as a dynamic interface between the peripheral circulation and the central nervous system. Type IV collagen is one of four major components of the basement membrane maintaining the structural integrity of the BBB [3]. Proteins of the matrix metalloproteinase family are involved in the breakdown of extracellular matrix [4], participate in the process of injury and repair in tissue [5] and are implicated in the damage of the BBB [6]. Matrix metallopeptidase 9 (MMP-9), also known as 92 kDa type IV collagenase, has a major role in the GCIR injury via degrading type IV collagen to undermine the integrity of the BBB [7]. The undermined BBB may lead to increased brain permeability, brain edema formation, aggravated infarction and hemorrhagic transformation [8-10]. Tea polyphenols (TP), polyphenolic compounds in tea, especially the epicatechin (flavan-3-ol compound), have strong antioxidant capacity [11]. Previous studies revealed that TP inhibit inflammatory response and have neuroprotective effects after ischemia reperfusion injury [12-14]. We hypothesized that the neuroprotective effects of TP might be implicated in maintaining the integrity of the BBB. Thereupon we conducted this primary study to investigate the protective effect of TP on the BBB after GCIR and its possible mechanisms. Our results demonstrate that TP may reduce the MMP-9 mediated collagen IV degradation caused by GCIR to protect the integrity of the BBB.

Materials and methods

Animals

The Ethics Committee of the Second Affiliated Hospital of Medical School of Xi'an Jiaotong University approved animal use for this study (certification No. 0045639). Sprague Dawley (SD) rats (male/female 1:1 ratio, 300±25 g) were purchased from the Laboratory Animal Center in the Medical School of Xi'an Jiaotong University. They were randomly classified into sham-operation (Sham) group, GCIR group and TP group (n = 30 per group). All of them were housed under standardized conditions in plastic cages (light-dark cycle 12/12 h, temperature 22 ± 2°C, humidity 50 ± 10%), fed with a standard diet, and had free access to tap water. Animal care and treatment were conducted in conformity with institutional guidelines that are in compliance with international laws and politics.

GCIR model

A one-stage anterior approach for four-vessel occlusion (4VO) was used in the rats to construct the GCIR model. Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 mL/mg) and placed in the supine position. A rectal temperature probe was inserted and body temperature was monitored and maintained at 37.0°C using a heating pad. The processes of isolating bilateral common carotid arteries (CCAs) and bilateral vertebral arteries (VAs) and occluding these arteries with microvascular clips were performed as described previously [15]. The cerebral

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blood flow (CBF) in the right hemisphere was measured continuously with a laser Doppler flowmeter as described previously [16]. The arteries were allowed to reperfuse 15 minutes after occlusion. Rats with sham operation underwent the same procedures with the exception of microvascular clip application.

TP administration

TP (6.4%) was purchased from Sigma-Aldrich Company (St. Louis, MO, USA) and injected into the TP-treated group (200 mg/kg) from the tail vein 30 min before cerebral ischemia.

Inclusion criteria

After surgery (4VO or sham-operation) the rats who complied with our experimental requirements were used for the subsequent experiments. Our experimental requirements were as follow: 1) the rats regained consciousness and moved around soberly within 2 h post surgery; 2) the rats stayed alive until the subseqent experiment.

Brain tissue collection

After anesthetization, rats were perfusion-fixed with 10 U/mL heparin and then with 4% paraformaldehyde (in 0.1 mol/L phosphate buttered saline, pH 7.4). Brain tissues (1-4 mm posterior to the chiasma opticum) were quickly removed and further fixed in 4% paraformaldehyde for 12 hours at 4°C. Post-fixed brains were embedded in paraffin followed by preparation of 5-μm thick coronal sections using a microtome.

HE stain

The paraffin-embedded brain slices were deparaffinized with xylene and rehydrated in a decreased ethanol series of 100%–80% (v/v), followed by washing with water. The slices were stained with hematoxylin and eosin and then examined by light microscopy.

Measurement of brain water content

The rats were decapitated 24 h after reperfusion and part of their brains removed to determine the wet weight (WW). These same parts were then baked in the oven (70°C) for 72 h and weighed again to obtain the dry weight (DW). This was calculated using the mathematical formula of brain water content (BWC): BWC (%) = [1 – (DW/WW)].

Measurement of BBB permeability

The residual amount of Evan's blue dye (Sigma-Aldrich, St. Louis, MO, USA) in brain tissue was measured to evaluate BBB disruption quantitatively. 24 h after reperfusion, rats were administered intravenously with Evan's blue dye (3 ml/kg; 2% wt/vol in PBS) via the tail vein. 1 h later, they were anesthetized and their thoracic cavities opened to expose the heart. Cold PBS (250 ml) was rapidly perfused into the left ventricle and ascending aorta. Subsequently the rats were decapitated and their brains were removed, weighed, and homogenized. Dimethylformamide (4-fold of sample volume) was added to the brain samples, which were then incubated in a 50°C water bath for 72 hr and centrifuged for 10 min at 3000r/min. The supernatant was collected and the dye in the supernatant was detected by UV spectrophotometer (Long Nike instrument Co. Ltd, Shanghai, China) at 635nm. We obtained the content of Evans blue from comparison with a standard curve expressed as μg/g.

Immunohistochemistry

24 h after reperfusion, SABC immunohistochemical (IHC) staining was employed to analyze the expression of MMP-9 and collagen IV in cerebral tissue. The primary antibodies of MMP-9 and collagen IV were respectively Anti-MMP-9 Antibody (1:100; Abcam, UK) and Anti- collagen IV Antibody (1:100; Abcam, Cambridge, UK). The secondary antibody was biotinylated goat anti-rabbit IgG (1:1000-3000; Phoenix Biotech Co., Ltd., Beijing, China). After DAB coloration, the protein expressions of MMP-9 and collagen IV were analyzed using the Leica QWin image processing and analyzing system (Leica, Solms, Germany). We randomly selected 5 different high-power fields from each tissue slice (HPF, 10×40) that did not overlap each other. The number of positive cells was counted, and the average was considered as expression index.

Statistical analysis

All data were expressed as Mean ± SD. Group differences were analyzed with one-way analysis of variance (ANOVA) and pairwise comparison with Student-Newman-Keuls-q test (SNK-q). Statistical significance was considered at $P<0.05$.

Results

Using a laser Doppler flowmeter it was recorded that the CBF was decreased to 10% and 15% of the baseline during the 15 min of 4VO, and had a slight overflow immediately after the completion of declamping the arteries (Figure 1).

Of the 30 rats undergoing sham-operation, none were excluded based on the inclusion criteria. But of the 60 rats receiving 4VO, 5 rats were excluded due to 2 of the rats in the GCIR

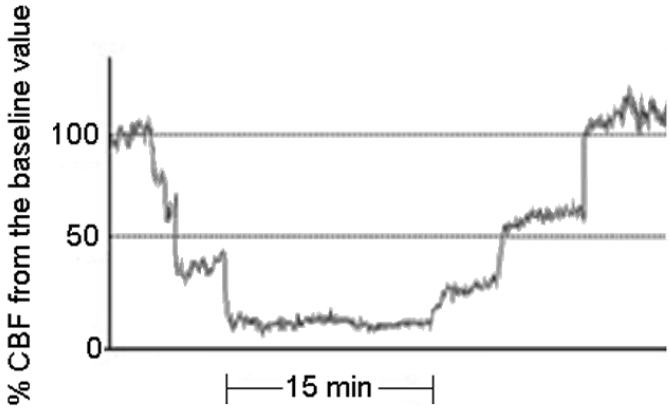

Figure 1. CBF evaluations are shown. The procedure of clamping arteries is: left VA, right VA, left CCA, right CCA, and that of declamping is reverse order.

group failing to regain consciousness within 2 h after surgery, 1 rat in the GCIR group and 2 rats in the TP group dying soon after the surgery.

Under microscope (HE staining, magnification ×400), the structure of the cerebral cortex of rats in the Sham group (Figure 2A) was clear, and the neurons appeared normal with orderly distribution. In contrast, rats in the GCIR group (Figure 2B) presented with a chaotic distribution of deformed neurons (including survived neurons, dead neurons and dissolved neurons). The TP group (Figure 2C) was observed to be better than the GCIR group in terms of the structure of the cerebral cortex, indicating that TP might protect the brain tissue against GCIR.

The measurement of BWC showed that compared to Sham rats, the BWC of the GCIR group and the TP group were obviously elevated ($P<0.05$), indicating that GCIR may damage the integrity of BBB and increase BBB permeability. However, the BWC of TP group was significantly lower than that of GCIR group ($P<0.05$) (Figure 3A), revealing that TP may protect the integrity of BBB to some extent. This was further verified by the analogous findings in the measurement of Evan's blue dye in the brain tissues (Figure 3B).

On the stained sections, the positive cells of MMP-9 expression that presented with yellow-brown were barely detected in the Sham group (Figure 4A) but distributed in the nuclear envelope of many cells in the GCIR group (Figure 4B). Compared to the GCIR group, the positive cells in the TP group (Figure 4C) were reduced. The comparison of the MMP-9 expression index between the two groups confirmed that this reduction was significant ($P<0.01$) (Figure 4D), suggesting that TP may inhibit the expression of MMP-9 in the brain tissues of rats with GCIR.

Collagen IV was also stained brown or dark yellow in IHC staining. Of the three groups, the collagen IV expression of the Sham group (Figure 5A) was the highest, followed by the TP group (Figure 5C) and lowest in the CIR group (Figure 5B). Pairwise comparison showed that the differences between the three groups were statistically significant ($P<0.01$) (Figure 5D). We noticed that collagen IV expression was opposite to that of MMP-9, namely down-regulated MMP-9 vs. up-regulated collagen IV or vice versa. These findings may suggest that TP attenuate the MMP-9 mediated degradation of collagen IV in the brain tissue of rats with GCIR.

Discussion

TP are chemical compounds such as flavanoids and tannins contained naturally in tea. Their potential medicinal value has been demonstrated by various studies. For example, TP have been shown to play a role in cancer prevention [17] and have successfully been used in the control of diabetes [18]. In addition, TP have anti-inflammatory effects [19] and

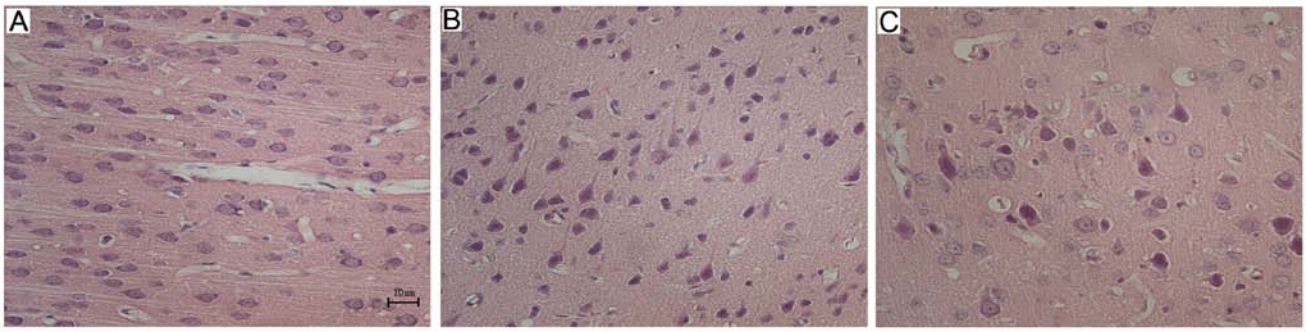

Figure 2. Morphology of cerebral cortex of Sham group (A), GCIR group (B), TP group (C) (n = 15 per group) are showed under microscope (HE staining, scale bar = 10 μm).

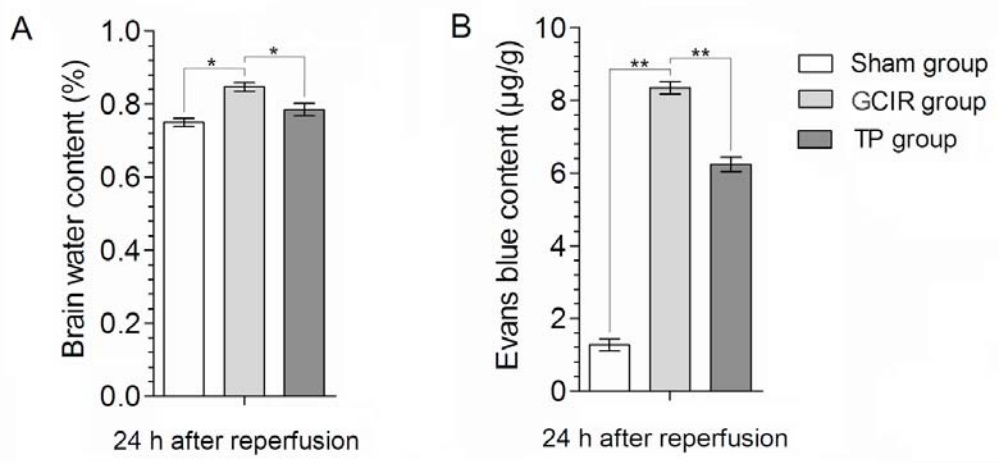

Figure 3. The brain water content of three groups (A) (n = 15 per group) and the residual amount of Evan's blue dye in the brain tissue of three groups (B) (n = 12 per group). * stands for $P < 0.05$, and ** $P < 0.01$.

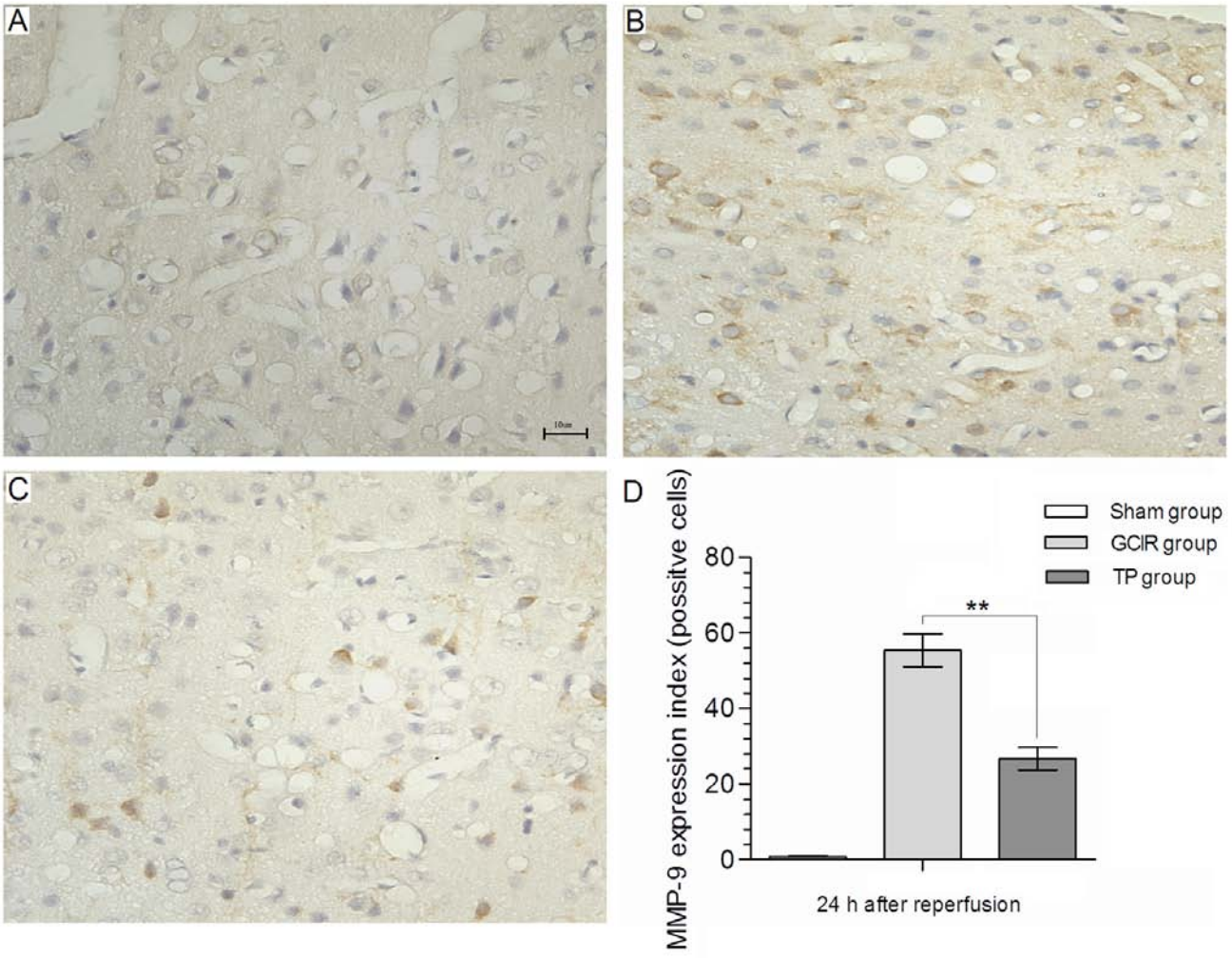

Figure 4. MMP-9 IHC expression in the cerebral tissue of Sham group (A), GCIR group (B) and TP group (C) (n = 15 per group) and their corresponding expression index (D). * stands for P< 0.05, and ** P < 0.01. Scale bar = 10 μm.

neuroprotective effects [13,14]. This current study further confirms the neuroprotective effects of TP from the perspective of BBB protection. Importantly this study reveals that TP may reduce the MMP-9 mediated degradation of collagen IV in the ischemic BBB microvasculature and this may be associated with the protective effect of TP on BBB.

Considering the advantages of one-stage anterior approach for 4VO in rat previously described by Yamaguchi et al [15], we adopted this method to construct the GCIR model. Our results show that the CBF goes down to 10% and 15% of the baseline after the completion of 4VO, which is basically in line with Yamaguchi's study (a decrease in CBF to 12% and 14% of the baseline). The complete occlusion time adopted by this study was 15 min rather than 10 or 30 min in Yamaguchi's study [15]. The reason for this is because we found that a 30 min occlusion time led to a relatively higher mortality rate in our pre-experiments, and that a 15 min occlusion time effectively reduced the mortality rate without changing the success of global cerebral ischemia.

Though reperfusion restores cerebral blood flow, it may induce secondary brain injury and cerebral edema [20]. In this study, we measured the BWC 24 h after reperfusion and found that it was increased compared to the Sham, suggesting that the rats with GCIR had cerebral edema. Cerebral edema is a complex and gradual process that results largely from impaired astrocytic cell volume regulation and permeability alterations in the cerebral microvasculature [21,22]. We conjectured that there must have been an injury in the integrity of the BBB and this injury might lead to an alteration in the permeability of the BBB. Thereupon we detected the permeability of the BBB by measuring the residual amount of Evan's blue dye in the brain tissue 24 h after reperfusion and confirmed that the permeability was obviously increased, indicating that an injury does exist in the integrity of BBB of rats with GCIR. It is noteworthy that all the rats with GCIR have a significant amelioration in cerebral edema after treatment with TP suggesting TP has a certain neuroprotective effect on the integrity of the BBB.

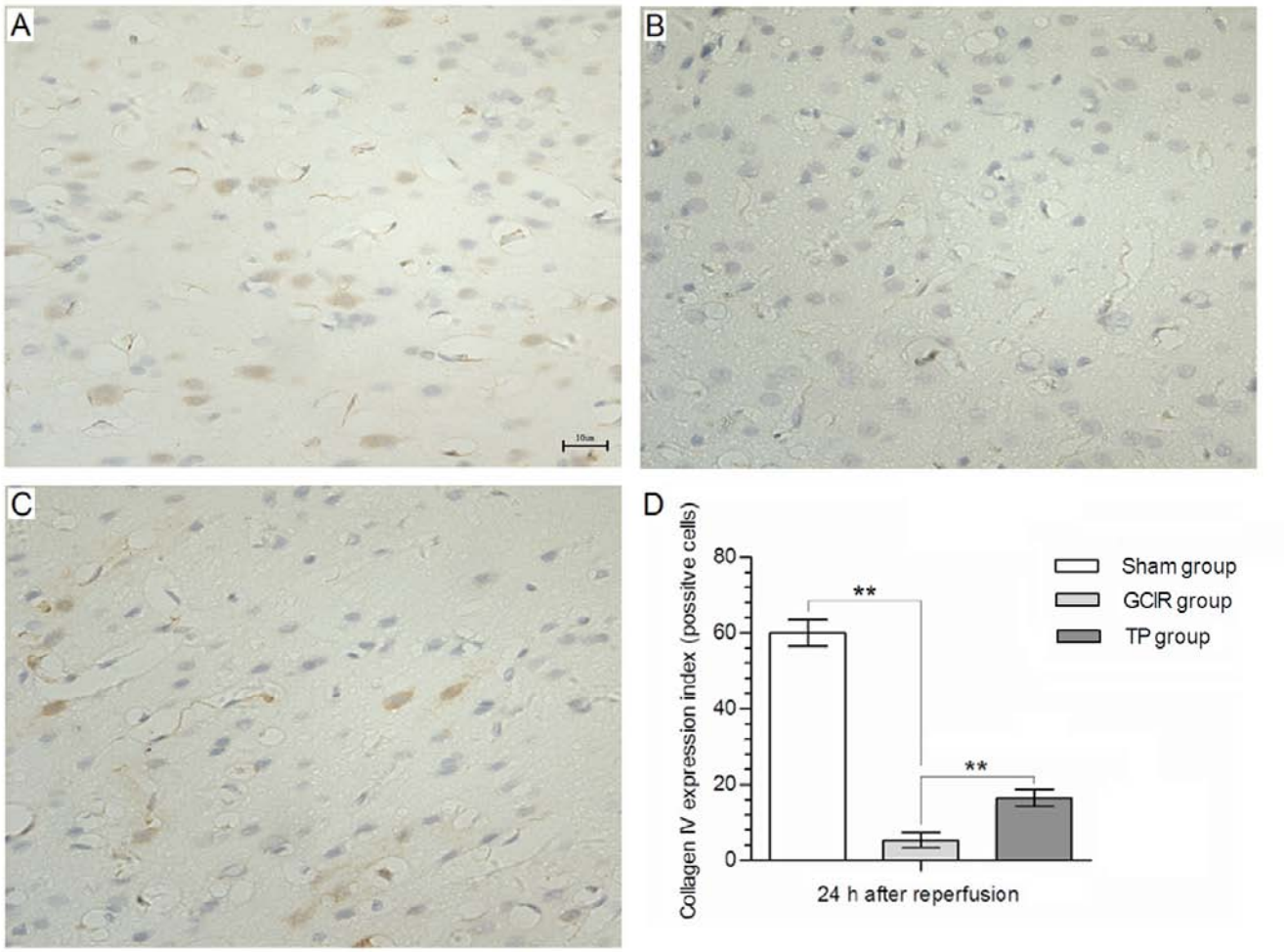

Figure 5. Collagen IV protein expression in the cerebral tissue of Sham group (A), GCIR group (B) and TP group (C) (n = 15 per group) and their corresponding expression index (D). * stands for $P < 0.05$, and ** $P < 0.01$. Scale bar = 10 μm.

We speculated that collagen IV and MMP-9 are involved in the neuroprotective capabilities of TP. Collagen IV is a major component of the basement membrane of the BBB and its degradation may lead to the disruption of integrity of the BBB and an increase in the permeability of the BBB. Collagen IV may further affect the outflow of water and neutrophils from vasculature, and influence the production of cerebral edema [8]. The major function of MMP-9 is to degrade collagen IV to lead to the breakdown of the BBB [7]. Normally there is no, or very low, expression of MMP-9 in brain tissue [23]. However, when the cerebral ischemia occurs, the ischemic brain tissues may produce active MMP-9, leading to an increase of MMP-9 expression in brain tissue [7]. The increased MMP-9 is bound to degrade the collagen IV, causing a decrease in collagen IV expression. This was proven by the IHC findings in the GCIR group of this study. In addition, this study finds that TP can effectively inhibit the expression of MMP-9 in the brain tissue of rats with GCIR. Prior studies have confirmed that MMP-9 activity is closely related to the inflammatory response [24] whereas, in contrast, TP have anti-inflammatory effects [19]. Perhaps the inhibitory effect of TP on MMP-9 is derived from its anti-inflammatory effect, which needs further investigation.

The basement membrane has four major components, collagen IV, laminin, nidogen/entactin and perlecan. Collagen IV and laminin individually self-assemble into suprastructures, and both networks are crucial for basement membrane stability. Nidogen/entactin and perlecan bridge the laminin and type IV collagen networks, increase their stability, and influence the structural integrity of the basement membrane [3]. A limitation of this study may be that, except for collagen IV, this study did not investigate the alterations of the other three basement membrane components in ischemic/reperfusion brain tissue. Another limitation may arise from the arbitrary sampling of region of interest on IHC stainings when quantifying the protein expressions, because this method is relatively prone to lead to

observer-induced bias. And due to absence of a stereologic approach, the obtained data should be considered as reflection of local apparent cellular densities rather than as estimation of the actual density or number of cells in the whole region of interest.

Conclusion

After careful consideration of all above mentioned points, we believe that TP may reduce the MMP-9 mediated collagen IV degradation caused by GCIR to protect the integrity of BBB, and that the potential medical value of TP deserves further experimental investigation.

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