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**ORIGINAL ARTICLE**

# Neuroprotective Effects of Grape Seed Procyanidin Extract on Ischemia-Reperfusion Brain Injury△

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**Key words:** grape seed procyanidin extract; oxidative stress; neuroprotection; ischemia-reperfusion injury

**Objective** Oxidative stress (OS) plays a crucial role in ischemic stroke. Grape seed procyanidin extract (GSPE) was reported to be a critical regulator of OS. We hypothesized that GSPE might also be protective in ischemia-reperfusion brain injury. This study aimed to explore whether GSPE administration can protect mice from ischemia-reperfusion brain injury.

**Methods** Transient middle cerebral artery occlusion (MCAO) was conducted followed by reperfusion for 24 hours to make ischemia-reperfusion brain injury in mice that received GSPE (MCAOG, n=60) or normal saline (MCAONS, n=60). Sham-operated mice (GSPE group and normal saline group) were set as controls. The neurological severity score (NSS) was used to evaluate neural function impairment 1 hour, 24 hour, 3 days and 7 days after MCAO. Mice underwent brain T2WI imaging with a 3T animal MRI scanner 24 hours after reperfusion, and the stroke volume of brains were calculated according to abnormal signal intensity. Immunohistopathological analysis of brain tissues at 24 h after reperfusion was performed for neuronal nuclear antigen (NeuN), CD34, Bcl-2, and Bax. Glutathione peroxidation (GSH-Px) activity and the level of malonaldehyde (MDA) of brain tissue were also examined. The above indexes were compared among the groups statistically.

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**Results** Significant functional improvement was observed 24 hours after MCAO in MCAOG group compared to MCAONS group ($P<0.05$). MCAOG group had smaller cerebral stroke volume ($22.46 \pm 11.45$ $mm^3$ *vs.* $47.84 \pm 9.06$ $mm^3$, $P<0.05$) than MCAONS group 24 hours after MCAO. More mature NeuN-immunoreactive neurons and more CD34-positive cells in peri-infarct zones were observed in brain tissue of MCAOG mice 24 h after MCAO than that of MCAONS mice (both $P<0.05$). MCAONS mice had significantly higher number of Bax-positive cells in brain tissue than MCAOG ($P<0.05$). The mean MDA level was significantly lower ($P<0.05$) and the GSH-Px activity was significantly higher ($P<0.05$) in brains of MCAOG mice compared to those of MCAONS mice.

**Conclusion** GSPE administration protects mice from ischemia-reperfusion brain injury through attenuating oxidative stress and apoptosis, promoting angiogenesis, and activating antioxidant enzyme GSH-Px. GSPE may represent a new therapeutical direction for the treatment of ischemia-reperfusion brain injury.

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STROKE is one of the most leading global causes of mortality in the world.[1] Even in patients with the same ischemia degrees, a large variability in neuronal functional recoveries exists. Therefore, it is vital to illuminate exact molecular biological mechanisms underlying the pathogenesis of ischemia-reperfusion brain injury and to explore more effective therapies.

Oxidative stress (OS) plays an important role in cardia-cerebrovascular diseases.[2] Many human and animal researches demonstrated a correlation between ischemia-reperfusion brain injury and increased systemic and local OS.[2, 3] Targeting OS in either primary ischemic-insult or the following reperfusion-injury, a lot of pharmacological drugs, particularly natural products derived from herbal medicine, are indicated to possess neuro-protective effect against ischemia-reperfusion brain injury.[4]

Grape seed polyphenol extracts (GSPEs) have strong anti-oxidant effects and can protect neurons and glia during ischemic-injury. GSPE contains a high concentration of flavonoids, linoleic acid fatty acids, and phenolic procyanidins. It has been said that the oxidant-lowering effect of GSPE is approximately fifty times higher than that of vitamin E and vitamin C. In a study by Prior *et al*, a dramatically increased post-prandial antioxidant capacity was observed after the consumption of mixed grape powder.[5] Nevertheless, the exact effect of GSPE on ischemia-reperfusion brain injury remains not clear. In our study, we explored whether mice that received GSPE could be protected from ischemia reperfusion-induced and OS mediated brain damage and function impairments. GSPE's effects on mitochondrial signaling pathways, which are crucially involve in OS processes, were also investigated.

## MATERIALS AND METHODS

### Animal preparation

Thirteen to 15-week-old C57 male mice were fetched from the animal house of Peking Union Medical College (Beijing, China). The mice were fed at a standard temperature (24°C–26°C) and an appropriate humidity and natural dark-light cycles for one week before the procedures to let them acclimate. All mice had free accesses to fresh food and water, and received humanized treatments according to the European Community guidelines for conducting experiments on animals.

### Experimental grouping design

To evaluate the roles of GSPE in protecting from ischemia-reperfusion brain damage, a transient MCAO surgery[6] was done on both GSPE and normal saline treated mice to induce a focal ischemic stroke. Following a midline cervical incision, a 6-0 silicone-coated filament was introduced into the left common carotid artery and advanced into the internal carotid artery (ICA) for 9-12 mm from the common carotid bifurcation. The thread was left in place for 60 min to block the blood flow, and then removed to allow reperfusion.

The animals were divided randomly into four groups as follows: (a) sham-operated GSPE (SOG) group: pretreated with GSPE 50 mg/kg by intraperitoneal injection per day for 2 weeks. Procedures of MACO model were performed, except that the silicone-coated filament was advanced into the ICA for 5 mm from the common carotid bifurcation without interruption of cerebral blood flow in the middle cerebral artery; (b) sham-operated normal saline (SONS) group: pre-treated with normal saline-water, and subjected to sham operational procedures; (c) MCAO GSPE (MCAOG) group: pretreated with GSPE 50 mg/kg by intra-peritoneal injections per day for 2 weeks, and then subjected to MCAO procedures (d) MCAO normal saline (MCAONS) group: pretreated with normal saline and subjected to MCAO procedures. The neuroprotective effect of GSPE was assessed using behavioral and histological techniques as described below. There were 60 mice in each group. Among the 60 mice, there were 15 for neurological deficit

measurement (1h, 24h, 1d, and 7d), 15 for MR imaging and immunohistochemical examinations at 24 h after MCAO, 15 for MDA measurement at 1h after MCAO, and 15 for MDA and GSP-Px measurement at 24 h after MCAO .

### Neurological deficit measurement

Neurological severity score (NSS) [7] evaluations were performed blindly 1 hour, 24 hour, 3 days and 7 days after MCAO. Neural functions were graded as 0 to 18, where grade 0 represents normal, and grade 18 represents maximal deficit. Since the mNSS showed an abnormal distribution, we used median and inter-quartile range to describe the data and the rank-sum test to compare the data.

### MR imaging and measurement of infarct volume

Mice were anesthetized with 10% chloral hydrate and underwent brain MRI scans using a 3T animal MRI-scanner (PharmaScan®, Bruker BioSpin, USA) 24 hours after reperfusion.[8,9] The mice were prostrated on a customer-made holder with strapping to minimize head motions. Coronal T2WI acquisitions were conducted from 2mm anterior corpus callosum to the end of the cerebrum with parameters as following: field of view (FOV)=2.5 cm × 2.5 cm, slice thickness=1.0 mm, echo time (TE) =20 ms, repetition time (TR) =11189 ms, and matrix size =128× 128. Stroke volumes (volumes of infarcted areas) were calculated automatically by image post-processing software (Brukey BioSpin) through regions of interest (ROI) method for the abnormal signal area of each involved slice.

### Histopathological analysis

24 hours after MCAO, mice (15 per group) were anesthetized with 4% isoflurane. Brains were excised after sacrifice. Paraffin sections (30 μm thick) were cut in compliance with general methods. The procedures were the same for all the 4 groups.

#### *NeuN and CD34*

Paraffin sections were stained with hematoxylin and eosin and antibodies to neuronal nuclear antigen NeuN（1:500; Abcam) and CD34 (1:500; Beyotime). NeuN was adopted to estimate the numbers of remnant mature neurons in peri-infarct zones.[10] CD34 was stained to estimate the numbers of microvessels in the ischemic boundary zone. [11] Secondary antibodie's visualization was performed using the ImmPRESS Universal (mouse/rabbit) Ig Kit (Vector Laboratories, Burlingame, CA, USA). All sections were examined under a light microscope. The positive cells in the peri-infarct region were counted with a 20× objective. In each slice only the area with the densest positive cells was chosen for counting.

#### *Bax/Bcl-2 staining*

To evaluate whether GSPE could attenuate cells apoptosis, sections were stained with antibodies against Bcl-2 (1:200; Sigma) and Bax (1:200; Sigma) over night at 4°C. After washing, the sections were incubated using the secondary antibody linked to horseradish peroxidase for half an hour. Cells that displayed brown precipitations were regarded as positive for Bcl-2 or Bax expressions.

### Determination of GSH-Px activities and MDA expressions

The level of malonaldehyde (MDA) and the activity of glutathione-peroxidase (GSH-Px) in brain tissue were assessed according to the instruction of commercial kits. Briefly, the brain tissue was homogenized in ice-cold 0.05 M PBS, and the homogenates were then filtered and centrifuged using a refrigerated centrifuge at 4°C. Then the supernatant was used to determin the enzyme activities by a microplate reader (Synergy TM, BioTek Instruments, USA). The protein expression of brain tissues was tested *via* Coomassie-Brilliant-Blue G-250 method with bovine serum albumin. The MDA level was shown in the form of nmol/L, and the GSH-Px activity in the form of U/mg protein.

### Statistical analysis

Data were given in the form of mean ± standard error of the mean (SEM). Since data of NSS showed an abnormal distribution (confirmed by the normality test), we used median and inter-quartile range to describe, and rank-sum test to compare the data. Statistical analysis was performed by 2-way analyses of variance (ANOVA) with post hoc multiple comparisons by virtue of Bonferroni correction, rank sum test, or unpaired/paired *t*-tests in a two-tailed way using SPSS Statistics (version 20.0) and GraphPad Prism software (version 6.0).[12] $P<0.05$ represents significant difference.

## RESULTS

### GSPE improves neurological function following MCAO

The median NSS with percentiles (5% to 95%) for MCAONS group ($n=15$) and MCAOG ($n=15$) group at 1 hour, 24 hours, 3 days, and 7 days were shown in Fig. 1. Both MCAONS and MCAOG mice exhibited marked coordination dysfunction at 1 hour after MCAO, with a median score of 15 and 14 respectively ($P=0.738$). Significant functional improvement was observed in MCAOG group at 24 hours after MCAO. The NSS decreased to a median score of 14 (quartile: 3.5) in MCAONS group and 11 (quartile: 3) in MCAOG group at 24 hours after MCAO (Mann-Whitney $U=59.0$; $P=0.026$;) and continuously decreased to 10

(quartile:2.5) in MCAONS group and 5 (quartile: 2.5) in MCAOG group respectively at 7 days (Mann-Whitney $U$=60.50; $P$=0.030), suggesting that GSPE administration did have neurotrophic effects on the ischemia-reperfusion injured brain. Both SONS and SOG mice did not exhibit any neurological deficit at the above time points (data not shown).

**Decreased brain infarction after ischemia-reperfusion injury in MCAOG mice**

Following 1 hour of blood flow interruption and 24 hours of reperfusion,[13] both MCAOG and MCAONS mice underwent a comparable weight loss (data not shown). Morphometrical analysis, based on brain MR Imaging,[8] revealed a significantly smaller stroke volume in MCAOG mice compared with MCAONS mice (MCAOG 22.46±11.45 $mm^3$ *vs.* MCAONS 47.84±9.06 $mm^3$, $P$<0.001) (Fig. 2). There were no abnormal signal intensities detected on T2WI in SOG mice and SONS mice.

**Neuroprotection and angiogenesis assessment by immunohistochemistry**

NeuN immunohistochemistry staining[10,11,14] showed that 24 h after reperfusion, the number of mature NeuN immunoreactive neurons in brain tissue of mice was higher in MCAOG group ($n$=15) than that in MCAONS group ($n$=15) (156.95±37.22 *vs*. 111.25±34.49, $P$= 0.0003) (Fig. 3 A-C). There was no significant difference in NeuN levels between sham-operation mice (SONS and SOG, $n$=15) and MCAOG mice ($P$=0.077). CD34 is an established marker of microvessel proliferation. There were more CD34-positive cells in the cortical peri-infarct zones of MCAOG mice than MCAONS mice (129.05 ± 36.94 *vs*. 104.00 ± 31.29, $P$= 0.0262) (Fig. 3 D-F). CD34 expression in brain tissue was not seen in either SONS group or SOG group (data not shown).

**Cell apoptosis assessed by immunohistochemistry**

Immunohistochemical staining for the proapoptotic protein Bax and antiapoptotic protein Bcl-2 was conducted 24 h after MCAO.[15] The number of Bax-positive cells in MCAONS was significantly higher than that in MCAOG (56.75±14.66 vs. 46.15±15.15; $P$= 0.0304) (Fig. 4 A-C), demonstrating that GSPE administration reduced the expression of Bax and inhibited the increase of Bax expression. Additionally, the number of Bcl-2-positive cells was significantly higher in MCAOG mice compared with MCAONS mice (53.15±11.88 *vs*. 35.15±13.62, $P$<0.0001) (Fig.4 D-F). No expression of Bax or Bcl-2 was detected in either SONS group or SOG group (data not shown). The results indicated that GSPE might protect the cells *via* up-regulation of anti-apoptotic proteins and down-regulation of pro-apoptotic proteins.

**GSPE attenuates OS and antioxidant enzyme**

Both MCAONS and MCAOG mice have shown significantly increased MDA compared to the sham groups (both $P$< 0.0001); however, the mean MDA level of the brains in the MCAOG group was significantly lower compared to that of the MCAONS group at the time points of 1h (MCAOG 2.97± 0.26 nmol/L *vs.* MCAONS 3.33±0.23 nmol/L, $P$=0.0419) and 24 h (MCAOG 2.54±0.19 nmol/L *vs.* MCAONS 2.70±0.23 nmol/L, $P$=0.0092) after reperfusion. There was no difference of MDA expressions between SONS group (2.20± 0.21 nmol/L) and SOG group (2.17±0.18 nmol/L) ($P$= 0.643 5) (Fig. 5). The activity of GSH-Px in the brains of MCAONS group (38.76± 4.04 U/mg) was significantly lower than that of MCAOG group (43.16±2.04 U/mg) ($P$=0.0001) (Fig. 6).

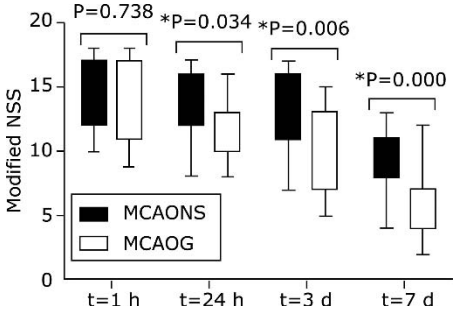


**Figure 1.** The neurological severity scores (NSS) for MCAONS mice and MCAOG mice at 1 h, 24 h, 3 d and 7 d after MCAO. Significant differences of NSS were observed at 24h, 3d, and 7d between group MCAONS and MCAOG. *$P$<0.05.

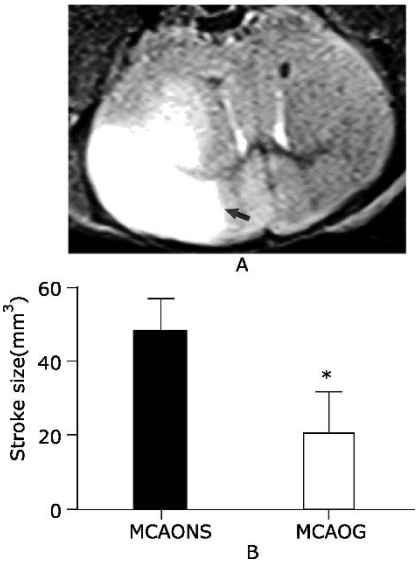


**Figure 2.** Stroke area after ischemia-reperfusion brain injury was measured by brain MRI (T2WI) 24 h after MCAO. A. High signal intensity was detected on T2WI in brain tissue of MCAOG mice, indicating cerebral infarction (arrow). B. Bar graph showing the mean stroke volume in MCAOG was significantly smaller than that of MCAONS mice. *$P$<0.001.

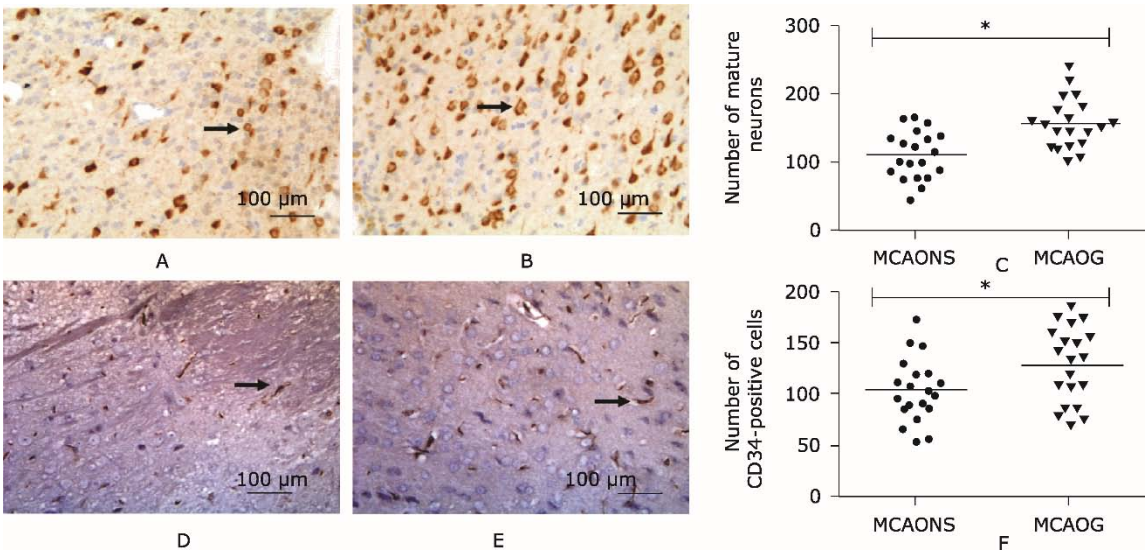


**Figure 3.** Comparison of NeuN and CD34 expression in the brain tissue of MCAONS and MCAOG group 24 hours after reperfusion by immunohistochemistry. A. Immunohistochemical staining of brain NeuN expression in MCAONS group (20×), and B. in MCAOG group (20×); C. The counting of mature NeuN immunoreactive neurons in MCAONS group was significantly less than that in the MCAOG group, $*P<0.05$; D. Immunohistochemical staining of brain CD 34 expression in the MCAONS group (20×), and E. in MCAOG group (20×); F. The counting of CD34-immunoreactive cells in the MCAONS group was significantly less than that in MCAOG group, $^{*}P<0.05$. Black arrows: positive staining.

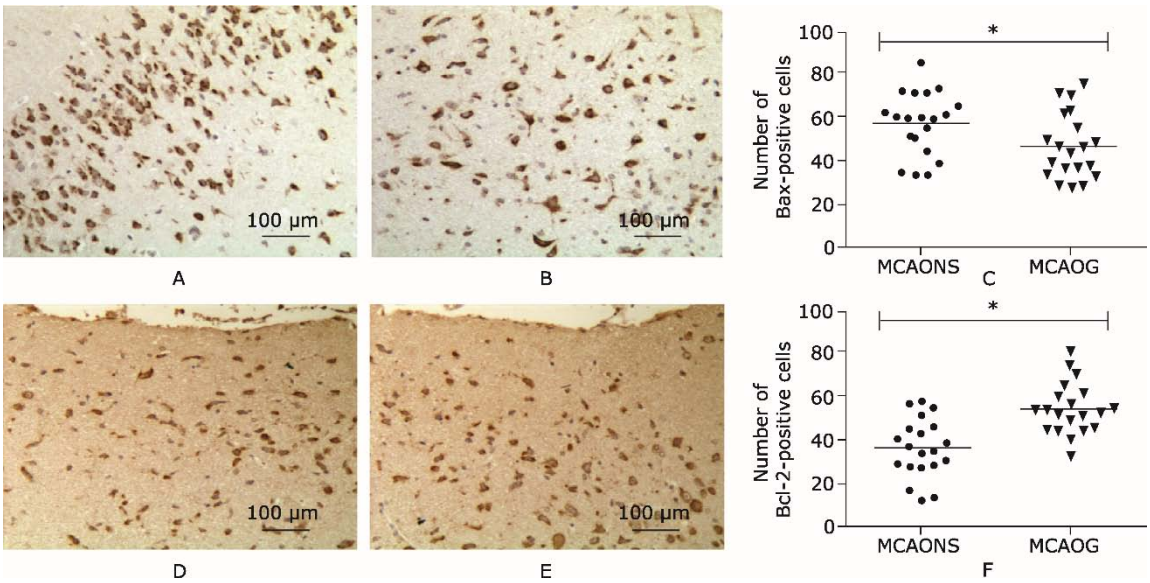


**Figure 4.** Comparison of Bax and Bcl-2 expression in the brain tissue of mice between MCAONA and MCAOG group. A. Immunostaining of the Bax-positive cells in MCAONS group (20×) and B. in MCAOG group (20×); C. The number of Bax-positive cells was significantly lower in MCAOG compared to MCAONS, $^{*}P<0.05$; D. Immunostaining of Bcl-2 positive cells in MCAONS group (20×) and E. in MCAOG group (20×); F. The number of Bcl-2 positive cells was significantly higher in MCAOG group compared to MCAONS group, $^{*}P<0.05$.

## DISCUSSION

GSPE is one of the potent free radical scavengers and is more powerful than vitamin E.[16] Previous researches have demonstrated that GSPE might attenuate inflammation and OS.[17] The number of literatures related to garlic or GSPE increases with years, most of which are from North America, Europe, East Asia and South Asia. Chen *et al.* found that GSPE has protective effect against renal OS induced by cadmium, and could serve as a natural agent against cadmium-poisonings.[4]

GSPE has unique advantages, though many medications have been shown to have anti-oxidant activities to

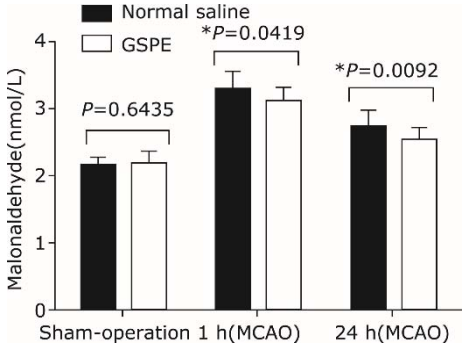


**Figure 5.** Comparison of MDA in brain tissues between the groups with pretreatment of GSPE and Normal saline. The MDA levels in brain tissues of MCAOG group 1 hour and 24 hours after reperfusion were significantly lower than those of MCAONS group ($^*P<0.05$ for both). There was no difference between SONS group and SOG group ($P>0.05$).

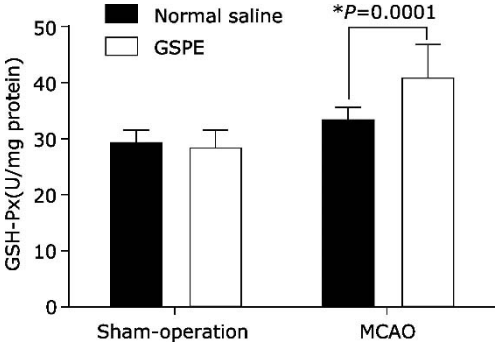


**Figure 6.** Comparison of GSH-Px in brain tissues between the groups pretreated with GSPE and normal saline. The activity of GSH-Px in the brains of MCAONS group was lower than that of MCAOG group.$^*P<0.05$.

protect against ischemia–reperfusion injury. GSPE showed concentration-dependent strong scavenging abilities against hydroxyl, peroxyl radicals, and superoxide anion.[18] In many countries, like Korea, Japan, and the United States of America, GSPE is used as an important nutritional supplement.[19] Our study revealed that GSPE administration protected mice from ischemia-reperfusion brain injury and consequent brain function impairments. This effect was paralleled by a reduced production of free radicals and inflammatory factors, an attenuated apoptosis, increased activities of anti-oxidant enzymes, including GSH-Px, and increased angiogenesis.

Transient MCAO is a well-established model of stroke,[20] and has been shown to be a reproducible and reliable rodent model of intracranial ischemia in humans for cognitive and sensorimotor deficits.[13] In the present study, this model induced sizeable strokes and neurological deficits. Encouragingly, GSPE administration protected mice from ischemia-reperfusion induced brain injury. After 1 h and 24 h of reperfusion, MCAOG displayed an approximately 50% reduction of cerebral infarction volume as shown by MRI, compared with MCAONS mice. The results of immunostaining of NeuN showed there were more neurons in MCAOG mice than in MCAONS mice, suggesting that mice received GSPE had more survived cells in the peri-infarct zones. Valuation of the neurological impairment 1 hour after MCAO displayed a similar degree in both MCAOG and MCAONS mice; nevertheless, at 24 hour after MCAO, MCAOG mice displayed an obvious less impairment in neurological functions compared with MCAONS mice. These findings have important clinical significance for translational medicine, because ischemia-reperfusion injuries are very common complications in ischemic-stroke patients who receive thrombolysis treatment.[21]

Increased OS intensity is widely considered as a key mediator of MCAO-induced brain injury. MDA is a biomarker for lipid peroxidation.[21, 22] Herein, we found that MCAONS mice displayed an increased MDA production in brain tissues compared with SONS mice, demonstrating that ischemia-reperfusion injury indeed results in OS. Interestingly, brain tissue of MCAOG mice displayed much lower levels of MDA production compared with MCAONS mice, suggesting that GSPE administration was importantly involved in the pathophysiological process.

CD34 is a recognized biomarker for the density of microvessels.[11] The results of immunostaining of CD34 showed there were more CD34-positive cells in the peri-infarct zone of MCAOG mice than MCAONS mice, suggesting GSPE may improve perfusion through the promotion of endothelial cell survival and the induction of neoangiogenesis. We also explored the apoptosis degree in peri-infarct zones *via* bax and bcl-2 staining. The results indicated that GSPE's anti-apoptosis effects were associated with up-regulating anti-apoptotic proteins (Bcl-2 positive) and down-regulating pro-apoptotic proteins (Bax positive).

GSH-Px is one of critical antioxidant enzymes and has the ability of scavenging endogenous free-radicals.[23] The GSH-Px activity of MCAOG group significantly increased compared to that of MCAONS group, suggesting that GSPE could improve the activities of antioxidant enzymes. The antioxidant activity of GSPE and its ability to inhibit cadmium-induced renal OS have already been observed.[4] Our results indicated that the GSPE antioxidant activity could also inhibit MCAO-induced brain OS and brain injuries.

In this study, the results of brain MRI, neurological

deficit assessments, immunohistochemical examinations, GSH-Px and MDA tests were consistent with each other. The mechanisms by which GSPE treatment prevents neuron losses may be correlated to the improvements of angiogenesis and blood flows, the reductions of OS and inflammation, and the repressions of apoptosis. All these indicated the potential therapeutic effects of GSPE in clinical treatments. Zhang *et al.* found that GSPE could decrease FoxO1 expression, improve granulosa-cell viabilities, upregulate LC3-II levels, and decrease granulosa-cell apoptosis degree. Under a condition of OS, GSPE could reverse nuclear localizations of FoxO1 and increase its levels in cytoplasm. In addition, FoxO1 knock-down could inhibit the protective-effects of GSPE.[16] Pallarès *et al.* found that GSPE had anti-inflammatory effects by reducing the pro-inflammatory marker NOx in red blood cells and plasma. Moreover, the high pharmacological dose also down-regulated the genes Il-6 and iNos; and the high nutritional dose could decrease the glutathione ratios. This further illustrate the antioxidant capability of GSPE.[24] However, the underlying molecular mechanisms of GSPE in anti-OS effects are still not clear enough, and need further explorations.

To conclude, the present research shows that GSPE exerts protective effects against ischemia-reperfusion brain injury via attenuating oxidative damages, and might attenuate the involved apoptosis through interfering with the expressions of Bcl-2 and Bax. The underlying mechanism may be associated with its antiapoptotic and antioxidant capacities. Nevertheless, the exact biomolecular mechanisms remain unclear and require further explorations.

***Conflict of Interest Statement***

*The authors have no conflicts of interest to disclose.*

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