Original Article

Ginsenoside Rd Attenuates DNA Damage by Increasing Expression of DNA Glycosylase Endonuclease VIII-like Proteins after Focal Cerebral Ischemia

Long-Xiu Yang[1,2], Xiao Zhang[1], Gang Zhao[1]

[1]Department of Neurology, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi 710032, China
[2]Department of Neurology, First Affiliated Hospital, Guangxi Medical University, Nanning, Guangxi 530021, China

Abstract

Background: Ginsenoside Rd (GSRd), one of the main active ingredients in traditional Chinese herbal *Panax* ginseng, has been found to have therapeutic effects on ischemic stroke. However, the molecular mechanisms of GSRd's neuroprotective function remain unclear. Ischemic stroke-induced oxidative stress results in DNA damage, which triggers cell death and contributes to poor prognosis. Oxidative DNA damage is primarily processed by the base excision repair (BER) pathway. Three of the five major DNA glycosylases that initiate the BER pathway in the event of DNA damage from oxidation are the endonuclease VIII-like (NEIL) proteins. This study aimed to investigate the effect of GSRd on the expression of DNA glycosylases NEILs in a rat model of focal cerebral ischemia.

Methods: NEIL expression patterns were evaluated by quantitative real-time polymerase chain reaction in both normal and middle cerebral artery occlusion (MCAO) rat models. Survival rate and Zea-Longa neurological scores were used to assess the effect of GSRd administration on MCAO rats. Mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damages were evaluated by the way of real-time analysis of mutation frequency. NEIL expressions were measured in both messenger RNA (mRNA) and protein levels by quantitative polymerase chain reaction and Western blotting analysis. Apoptosis level was quantitated by the expression of cleaved caspase-3 and terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling assay.

Results: We found that GSRd administration reduced mtDNA and nDNA damages, which contributed to an improvement in survival rate and neurological function; significantly up-regulated NEIL1 and NEIL3 expressions in both mRNA and protein levels of MCAO rats; and reduced cell apoptosis and the expression of cleaved caspase-3 in rats at 7 days after MCAO.

Conclusions: Our results indicated that the neuroprotective function of GSRd for acute ischemic stroke might be partially explained by the up-regulation of NEIL1 and NEIL3 expressions.

Key words: DNA Damage; DNA Glycosylase; Endonuclease VIII-like Proteins; Ginsenoside Rd; Ischemic Stroke

Introduction

Stroke, of which ischemic stroke is the most common type, remains one of the major causes of mortality and disability worldwide.[1] Among multiple molecular mechanisms involved in ischemic stroke, oxidative stress plays an important role in neuronal injury by causing damage to cellular proteins, lipids, and DNA.[2] Oxidative DNA damage can trigger dysfunction and death of brain neurons and eventually lead to poor outcomes.[3] Maintaining genome integrity of neurons after ischemia is considered to be an important strategy to improve the prognosis of ischemic strokes.[4] The predominant DNA repair pathway for oxidative DNA damage is base excision repair (BER).[5] BER is initiated by DNA glycosylases, which recognize and excise the damaged bases from the DNA.[6] The endonuclease VIII-like (NEIL) proteins, NEIL1, NEIL2, and NEIL3, are three of the five major DNA glycosylases that remove oxidative base lesions.[6] Previous data have indicated that *NEIL* gene knockout models exhibit genomic instability.

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.4103/0366-6999.187851

Address for correspondence: Prof. Gang Zhao, Department of Neurology, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi 710032, China
E-Mail: zhaogang@fmmu.edu.cn

Received: 04-04-2016 **Edited by:** Xin Chen
How to cite this article: Yang LX, Zhang X, Zhao G. Ginsenoside Rd Attenuates DNA Damage by Increasing Expression of DNA Glycosylase Endonuclease VIII-like Proteins after Focal Cerebral Ischemia. Chin Med J 2016;129:1955-62.

NEIL1 gene knockout mice, for example, showed an increased level of DNA damage compared to wild-type mice, and *NEIL2* gene knockout mice displayed an accumulation of oxidative genomic damage.[7,8] Evidence also suggested that NEILs may affect stroke prognosis. For instance, the *NEIL1* gene knockout mice with middle cerebral artery occlusion (MCAO) exhibited impaired memory retention, and *NEIL3* gene knockout mice showed poor outcomes after ischemic stroke, suggesting that NEILs might be essential elements for better outcomes.[7,9]

Panax ginseng is a well-known traditional herbal medicine which has been widely used in China for centuries, and ginsenoside Rd (GSRd) is one of its main active ingredients.[10] In our previous studies, GSRd has been shown to protect against ischemic cerebral damage via various mechanisms both *in vitro* and *in vivo*.[10] Furthermore, a randomized, double-blind, placebo-controlled, multicenter, phase II clinical trial, wherein 386 patients with acute ischemic stroke received a 14-day intravenous infusion of GSRd or placebo within 72 h after the onset of stroke, found that GSRd-treated patients showed significantly improved primary outcome over the control group.[11] The molecular mechanisms that underpin GSRd's neuroprotective function, however, have not yet been fully understood. Thus, as an extending study to further elucidate the underlying mechanisms, this study investigated the effect of Rd on the expression of DNA glycosylases NEILs in a rat model of focal cerebral ischemia.

Methods

Experimental animals and middle cerebral artery occlusion model

Male Sprague-Dawley rats, weighing 270–320 g, were provided by the Animal Center of the Fourth Military Medical University (Xi'an, China). Animal experiments were approved by the Ethics Committee for the Animal Care and Use Committee of the Fourth Military Medical University. A total of 96 rats were evenly and randomly allocated to four experimental groups: sham ($n = 24$), sham + GSRd ($n = 24$), MCAO ($n = 24$), and MCAO + GSRd ($n = 24$). MCAO was performed as described previously.[10] In brief, rats were anesthetized with 10% chloral hydrate by intraperitoneal injection (0.35 ml/100 g). First, the right common carotid artery was exposed and the external carotid artery was cut. Second, a ligature was applied for 2 h by passing a 4-0 monofilament with the rounded tip through the right external carotid artery and into the middle carotid artery. Body temperature was monitored with a thermostatically controlled heating blanket to maintain 37.0 ± 0.5°C. Sham-surgery rats were subjected to the same surgical procedures except that the monofilament was not advanced into the middle carotid artery. GSRd with a purity of 98% was obtained from Tai-He Biopharmaceutical Co. Ltd. (Guangzhou, China). Stock solutions were prepared in saline containing 10% of 1, 3-propanediol (v/v). For rats in the sham + GSRd and MCAO + GSRd groups, GSRd with a concentration of 30 mg/kg was applied intraperitoneally 1 h before MCAO and 10 $mg \cdot kg^{-1} \cdot d^{-1}$ until the rats were sacrificed. Rats in the sham and MCAO groups were intraperitoneally injected with the same amount of sterile saline water at the same intervals as the sham + GSRd and MCAO + GSRd groups.

Zea-Longa neurological deficit scores

The neurological scores were blindly assessed by two pretrained technicians independently at 2 h, 8 h, and then every 24 h up to 7 days after MCAO according to the Zea-Longa neurological deficit scores.[12] The Zea-Longa assessment criteria are as follows: score 0, normal, no neurological sign; score 1, cannot completely stretch contralateral forelimbs; score 2, contralateral circling when walking; score 3, contralateral fall over when walking; and score 4, cannot walk and lowering of consciousness. Rats were first evaluated 2 h after MCAO and the model was disqualified when it scored 0 or 1.

Quantitative real-time polymerase chain reaction

The total RNA was isolated using RNeasy kit (Qiagen, Germany) and RNA was reversely transcribed with high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). Subsequently, quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in a total volume of 20 μl, containing 11 μl of SYBR-Green I PCR master mix (Applied Biosystems, California, USA), 6 μl of cDNA (10 ng/μl), and 1 μl of 2.5 μmol/L forward and reverse primers, using a StepOnePlus Real-Time PCR Machine (Applied Biosystems) in standard cycling conditions. All Ct values were first normalized to reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and relative gene expression levels were calculated using the standard 2(–Delta Delta C (t)) method. The primers used in quantitative polymerase chain reaction were as follows: *NEIL1* forward 5'-TGGAGTATGAAGGGTTCAGAGAGA-3' and reverse 5'-GCCGATTCCATTGAAGAACCT-3'; *NEIL2* forward 5'-CAGTGCCCAGGCTGGTACTC-3' and reverse 5'-CTCGATGGAACTTTTCAGACAAGA-3'; and *NEIL3* forward 5'-GTGGAAAGCCAACAAAGAGT-3' and reverse 5'-TTTACGGCACCTGTAGAAGA-3'.

Western blotting analysis

After MCAO, the ipsilateral cortex and hippocampus were homogenized on ice in the radioimmunoprecipitation assay buffer (Sigma, USA) with 0.5 mmol/L phenylmethanesulfonyl fluoride (Sigma). Western blotting analysis was performed according to the study of Zhang *et al*.[10] The primary antibodies were NEIL1 antibody (1:100; Santa Cruz Biotechnology Inc., Texas, USA; sc-47611), NEIL2 antibody (1:100, Santa Cruz Biotechnology Inc., sc-135243), NEIL3 antibody (1:100, Santa Cruz Biotechnology Inc., sc-134835), and cleaved caspase-3 antibody (1:500; Cell Signaling Technology Inc., Beverly, Massachusetts, USA; Asp175). The secondary antibodies were anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody (1:5000, Cell Signaling Technology Inc., 7074), and donkey anti-goat IgG HRP antibody (1:5000; Abcam, Cambridge, USA; ab97110). Target protein signals were detected by

the chemiluminescence system (GE Healthcare, Amersham, UK), and ImageJ 1.41 (National institutes of health, Maryland, USA) was used for quantitative analysis. The β-actin (1:5000, Abcam, ab8227) or GAPDH (1:10,000, Abcam, ab8245) was used as a loading control.

Terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling staining

To assess the number of apoptotic cells, brain sections were stained with the *In Situ* Cell Death Detection Kit, Fluorescein (Roch Inc., Mannheim, Germany), according to manufacturer's instructions. Terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL)-positive cells in rat's ipsilateral cortices at 7 days after MCAO were observed through Zeiss LSM 510 Confocal Microscope (Carl Zeiss, Chicago, USA) with a 40× objective and counted by two independent investigators. The total number of TUNEL-positive cells was quantified in five randomly selected fields.

DNA damage quantification

DNA damage was evaluated with real-time analysis of mutation frequency (RAMF).[13] In brief, the genomic DNA was isolated with DNeasy blood and tissue kit (Qiagen, Germany) and 6 ng of DNA was added to either non-TaqI or TaqI containing SYBR Green PCR master mix. The primers to detect mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damage were as follows: 12S forward 5'-ACTCAAAGGACTTGGCGGTA-3' and reverse 5'-AGCCCATTTCTTCCCATTTC-3'; and NDUFA9 forward 5'-TGGTGACTCCTACCTGAAGC-3' and reverse 5'-TTCGGTCGTGAATTTTGTTT-3', respectively. The qRT-PCR program was initiated at 65°C for 15 min, followed by 94°C for 10 min, plus 40 cycles at 94°C for 15 s and 60°C for 1 min. DNA damage frequency was calculated as $2exp\text{-}(CT^{TaqI} - CT^{NT})$.

Statistical analysis

Each assay consisted of three independent experiments. Statistical analysis was performed using two-tailed Student's *t*-test. For multiple comparisons, Kruskal-Wallis test followed by Dunn's multiple comparison test was used. All data were analyzed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered statistically significant.

Results

Ginsenoside Rd improved the survival rate and neurological scores of rats after middle cerebral artery occlusion

We found that GSRd administration increased survival rate in the MCAO rat model [Figure 1a]. At the end of the study, 10 rats (out of 22) and 3 rats (out of 20) died in the MCAO and MCAO + GSRd groups, respectively. The survival rate of the MCAO + GSRd group (85%) was significantly improved over the control group (55%; $\chi^2 = 4.53$, $P = 0.03$). The effects of GSRd administration on neurological scores of the MCAO and MCAO + GSRd groups were evaluated according to the Zea-Longa neurological deficit scores. There were no significant differences at 2 h, 8 h, 1 day, and 2 days after MCAO. However, GSRd reduced the neurological deficit scores at 3–7 days after MCAO [Table 1 and Figure 1b].

Ginsenoside Rd decreased apoptosis in rat ipsilateral cortices at 7 days after middle cerebral artery occlusion

To investigate the effect of GSRd on apoptosis after MCAO, the cleaved caspase-3 was measured by Western blotting analysis [Figure 2a and 2b]. It was found that cleaved caspase-3 levels were significantly higher in the MCAO group and lowered by GSRd treatment. TUNEL staining was performed in rat's ipsilateral cortices at 7 days after MCAO [Figure 2c and 2d]. The TUNEL-positive cell count in the MCAO + GSRd group was significantly lower than that in the MCAO group, implying that GSRd decreased cell apoptosis in rat's ipsilateral cortices at 7 days after MCAO.

Ginsenoside Rd reduced mitochondrial DNA and nuclear DNA damage after middle cerebral artery occlusion

Using RAMF, the mtDNA and nDNA damages were evaluated and quantitated by RT-PCR in the MCAO and MCAO + GSRd groups [Figure 3]. At 3 and 7 days after MCAO, both mtDNA and nDNA damage levels were significantly increased in the MCAO and MCAO + GSRd groups. The mtDNA damage levels were lower in the MCAO + GSRd group than those in the MCAO group at both 3 and 7 days after MCAO. The nDNA damage levels were also lower in the MCAO + GSRd group versus the MCAO group at both 3 and 7 days after MCAO, but only with a significant difference at 7 days after MCAO.

Distribution patterns of *NEIL*s in brains of normal and middle cerebral artery occlusion rat

To evaluate the distribution patterns of *NEIL*s in normal rat brains, the expression levels of *NEIL*s were measured by qRT-PCR in different regions of untreated rat brains, including the cortex, hippocampus, brainstem, cerebellum, spinal cord, corpus striatum, and thalamus [Figure 4a].

Table 1: Zea-Longa neurological deficit scores of MCAO and MCAO + GSRd groups after GSRd administration

Time points	MCAO group		MCAO + GSRd group		*t*	*P*
	Numbers	Scores, mean ± SD	Numbers	Scores, mean ± SD		
2 h	22	2.8 ± 0.1	20	2.9 ± 0.3	0.23	0.82
8 h	22	2.2 ± 0.3	20	2.8 ± 0.3	1.83	0.08
1 days	18	2.2 ± 0.4	19	2.2 ± 0.1	0.25	0.80
2 days	17	2.6 ± 0.1	19	2.0 ± 0.9	2.00	0.07
3 days	15	2.9 ± 0.1	18	1.9 ± 0.1	4.59	<0.05
4 days	15	2.6 ± 0.1	18	1.8 ± 0.2	2.75	0.01
5 days	14	2.5 ± 0.2	18	1.7 ± 0.3	2.28	0.03
6 days	13	2.5 ± 0.1	17	1.7 ± 0.1	3.99	<0.01
7 days	12	2.4 ± 0.2	17	1.7 ± 0.1	3.37	<0.01

MCAO: Middle cerebral artery occlusion; GSRd: Ginsenoside Rd; SD: Standard deviation.

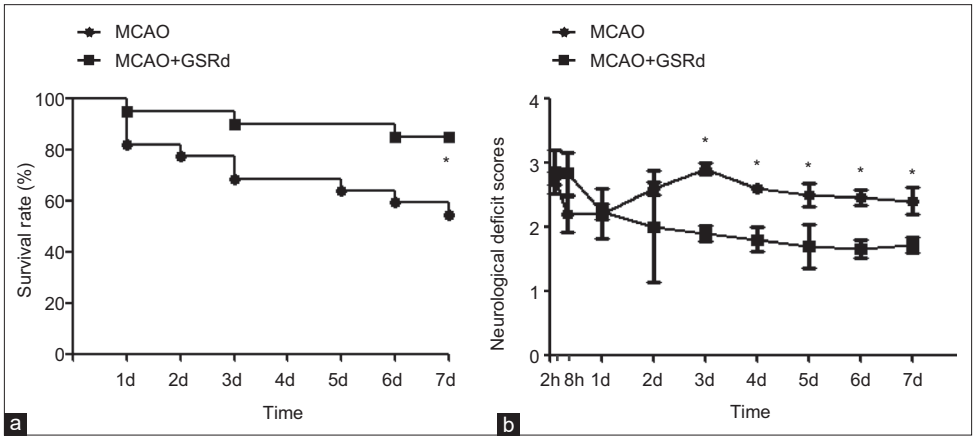

Figure 1: GSRd improved the survival rate and neurological scores of rats after MCAO. (a) The survival rates of the MCAO and MCAO + GSRd groups: MCAO group (n = 22) and MCAO + GSRd group (n = 20) were followed up for 7 days. *P = 0.033, compared with MCAO group. (b) The evaluation of neurological deficit scores: assessed at 2 h, 8 h, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, and 7 days according to Zea-Longa neurological deficit scores. Statistical differences were indicated as *P < 0.05, compared with MCAO + GSRd group. MCAO: Middle cerebral artery occlusion; GSRd: Ginsenoside Rd.

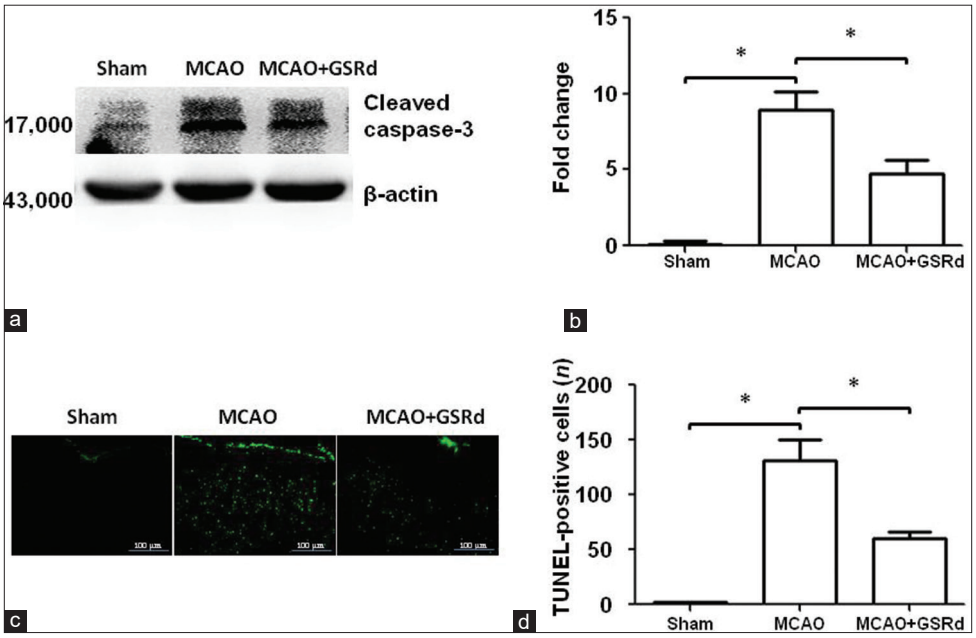

Figure 2: GSRd decreased apoptosis in rat's ipsilateral cortices at 7 days after MCAO. (a and b) The representative images and quantitative data analyses of Western blotting of cleaved caspase-3 at 7 days after MCAO. β-actin was used as a loading control. (c and d) The representative images and quantitative data analyses of TUNEL staining in rat's ipsilateral cortices at 7 days after MCAO. Data are presented as mean ± SD from 3 independent experiments (n = 3 biological replicates). *P < 0.05. Scale bar = 100 μm. MCAO: Middle cerebral artery occlusion; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling; SD: Standard deviation; GSRd: Ginsenoside Rd.

NEIL1 and *NEIL2* showed ubiquitous expression in all the regions measured. Compared with cortex, *NEIL1* was highly expressed in the hippocampus (t = 2.19, P = 0.04) and cerebellum (t = 2.59, P = 0.02); *NEIL2* showed a moderate expression increase in the corpus striatum (t = 2.54, P = 0.02); and *NEIL3* was dominantly expressed in the hippocampus (t = 2.29, P = 0.04).

The expression patterns of NEILs were further tested in samples from rat ipsilateral cortex at 12 h, 1 day, 3 days, and 7 days after MCAO [Figure 4b]. The expression of *NEIL1* increased gradually, peaking at 3 days after MCAO with an 87-fold increase over the sham group (t = 18.18, P < 0.01) and falling to a 41-fold increase over sham group (t = 2.67, P = 0.02). *NEIL2* showed an increasing trend after MCAO without significant difference among groups (P > 0.05). Similar to *NEIL2*, the expression of *NEIL3* also gradually increased without significant difference among groups for the first 3 days after MCAO, but increased significantly to 82-fold at 7 days after MCAO (t = 22.65, P < 0.01). In summary, *NEIL1* showed an early response, *NEIL3* showed a later response, and *NEIL2* had a less pronounced increase after MCAO. These results suggested that *NEIL*s responded and acted differently after acute ischemic stroke.

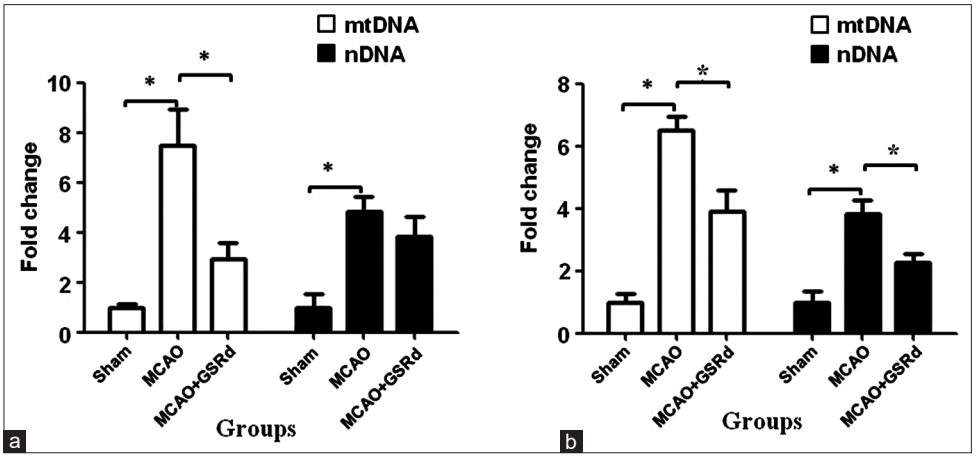

Figure 3: The effect of GSRd on mtDNA and nDNA damages at 3 days (a) and 7 days (b) after MCAO. Data are presented as mean ± SD from 3 independent experiments ($n = 6$ biological replicates). *$P < 0.01$. mtDNA: Mitochondrial DNA; nDNA: Nuclear DNA; MCAO: Middle cerebral artery occlusion; SD: Standard deviation; GSRd: Ginsenoside Rd.

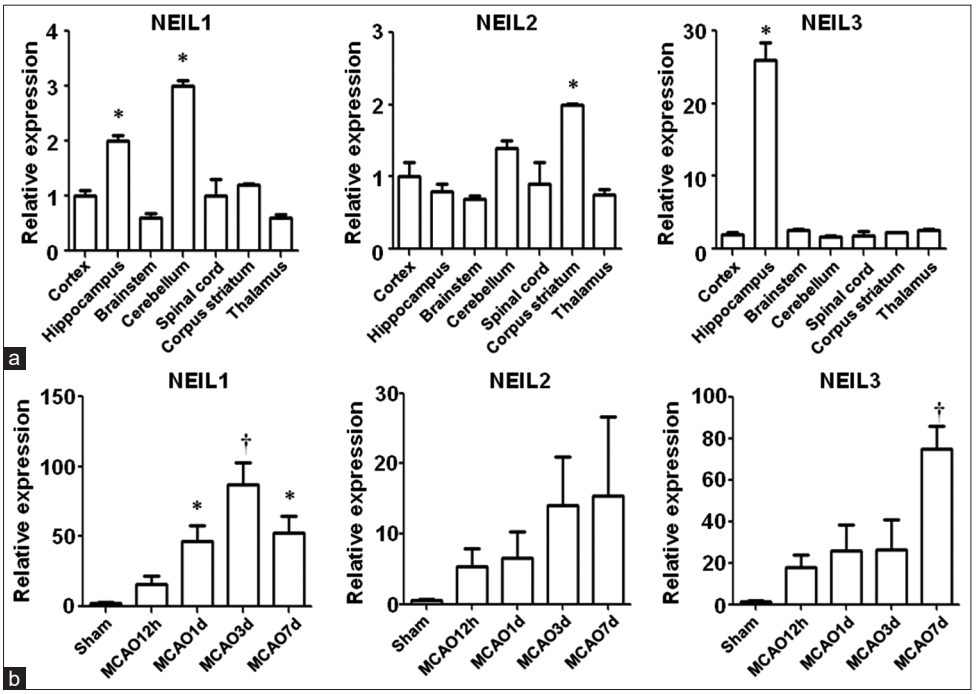

Figure 4: Regional expression analyses of *NEIL*s in brains of normal rat and MCAO rat by qRT-PCR. (a) The expression of *NEIL*s in normal rat brains. *$P < 0.05$, compared with cortex. (b) The distribution of *NEIL*s in MCAO rat brains. *$P < 0.05$ and †$P < 0.01$, versus sham group. Data are presented as mean ± SD from 3 independent experiments ($n = 3$ biological replicates). Data were normalized to *GAPDH* expression. *NEIL*: Endonuclease VIII-like; qRT-PCR: Quantitative real-time polymerase chain reaction; MCAO: Middle cerebral artery occlusion; SD: Standard deviation; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.

Ginsenoside Rd increased NEIL1 expression at 3 days and NEIL3 expression at 7 days after middle cerebral artery occlusion

To evaluate the effect of GSRd administration on the expression of *NEIL*s, both messenger RNA (mRNA) and protein levels of *NEIL* were measured in rat ipsilateral cortices and hippocampi at 3 days and 7 days after MCAO [Figure 5]. At 3 days after MCAO, the mRNA expressions of *NEIL*s were significantly increased in both cortex and hippocampus compared to the sham and sham + GSRd groups, with the exception of *NEIL2* [Figure 5a and 5b]. Only *NEIL1* expression was significantly increased in the MCAO + GSRd group over the MCAO group in the cortex [Figure 5a]. At 7 days after MCAO, *NEIL1* and *NEIL3* mRNA levels were higher in the MCAO and MCAO + GSRd groups in both cortex and hippocampus [Figure 5c and 5d]. However, only *NEIL3* expression increased in the MCAO + GSRd group over the MCAO group in both cortex and hippocampus [Figure 5c and 5d]. The changes in *NEIL* expressions were further confirmed at protein level. The results of Western blotting analysis showed that NEIL1 expression

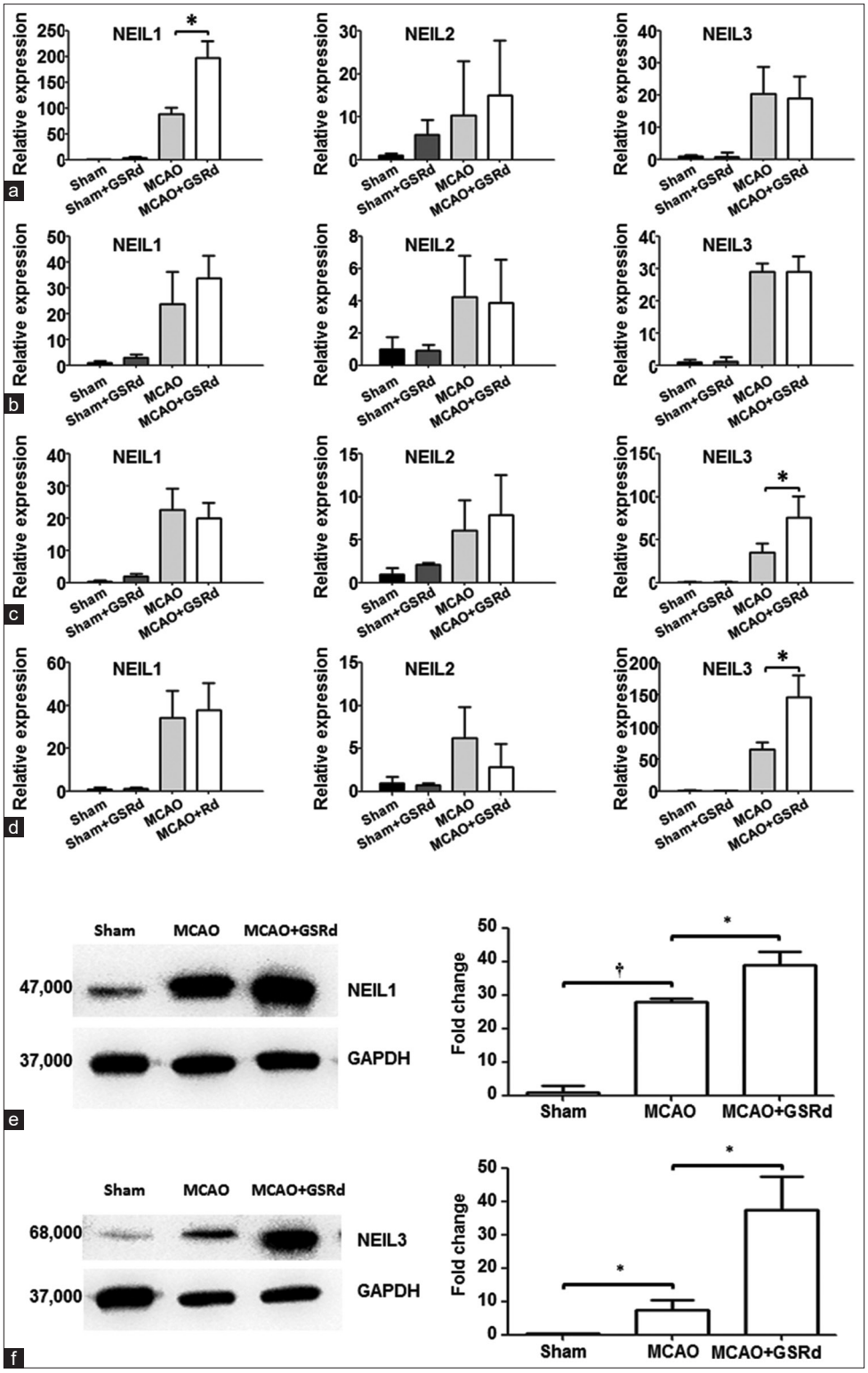

Figure 5: The effect of GSRd on the expression levels of *NEIL*s after MCAO. (a and b) The mRNA expression levels of *NEIL*s at 3 days after MCAO in rat ipsilateral cortices and hippocampi. (c and d) The mRNA expression levels of *NEIL*s at 7 days after MCAO in rat ipsilateral cortices and hippocampi. (e) Western blotting analysis of NEIL1 in rat ipsilateral cortices at 3 days after MCAO. (f) Western blotting analysis of NEIL3 in rat ipsilateral hippocampi at 7 days after MCAO. $^{*}P < 0.05$ and $^{\dagger}P < 0.01$. Data are mean ± SD from three independent experiments ($n = 6$ biological replicates). mRNA: Messenger RNA; NEIL: Endonuclease VIII-like; MCAO: Middle cerebral artery occlusion; GSRd: Ginsenoside Rd; SD: Standard deviation.

was significantly increased by GSRd administration at 3 days after MCAO in the cortex [Figure 5e], and NEIL3 expression was significantly increased by GSRd administration at 7 days after MCAO in the hippocampus [Figure 5f]. No significant difference in NEIL1 and NEIL3 protein levels was found between the MCAO and MCAO + GSRd groups in the rest of the comparisons and NEIL2 was not detected by Western blotting analysis.

Discussion

The previous studies showed that GSRd had a neuroprotective function in ischemic stroke.[10,11] In the present study, we investigated the effects of GSRd on the expression of DNA glycosylase NEILs in a rat MCAO model. Consistent with the previous findings, our results demonstrated that GSRd improved survival rate and neurological function. Moreover, we found that GSRd upregulated NEIL1 and NEIL3 expression levels. This suggested a correlation between the upregulation of NEIL1 and NEIL3 expression levels and a reduction in mtDNA and nDNA damage and, therefore, cell apoptosis.

Our previous work has revealed that GSRd is a neuroprotective agent through ameliorating oxidative stress after ischemic stroke.[14,15] Similar results were observed in another study wherein oxidative stress was suppressed by GSRd pretreatment in the dopamine-induced apoptosis PC12 cell model.[16] In seeking to further explore the neuroprotective mechanism of GSRd after ischemic stroke, we found that GSRd upregulated the expression of NEIL1 in the cortex at 3 days after MCAO and NEIL3 in the hippocampus at 7 days after MCAO in a rat model. However, we did not detect NEIL2 by Western blotting analysis, which may be induced by the relative low expression level of NEIL2 in rat brains. The differences in region and time course between NEIL1 and NEIL3 may be due to their different distributions in rat brains and NEILs responses after acute ischemic stroke. We also found that GSRd administration reduced the mtDNA and nDNA damages and expression of cleaved caspase-3. We proposed that by increasing NEIL1 and NEIL3 expressions, GSRd can enhance the BER pathway to reduce oxidative DNA damage, maintaining the genome integrity of neurons and subsequently decreasing apoptosis to improve the outcome of ischemic stroke in the rat model. This also indicated a strong association between the BER capacity and outcome of ischemic stroke. In line with our findings, other studies also found that enhancing the BER pathway can improve the recovery of ischemia and potentially be used as a neuroprotective strategy for ischemic stroke. Nicotinamide adenine dinucleotide (oxidized form; NAD^+) repletion was reported to confer neuroprotection against ischemic cell death in rat primary neuronal cultures subjected to oxygen-glucose deprivation by increasing BER activity.[17] Ebselen, a glutathione peroxidase mimic, was reported to enhance repair activity in the thalamus after focal cortical infarction in hypertensive rats by attenuating DNA damage through increased DNA glycosylase OGG1 expression, which initiated the BER pathway.[18]

Furthermore, we found that NEIL3 was dominantly expressed in rat hippocampi, one of the restricted neurogenesis regions in the adult brain, in both normal and MCAO rats. In line with our results, using *in situ* hybridization and Northern blotting, Rolseth *et al*.[19] found that NEIL3 showed expression preference in the stem cell harboring region, including the subventricular zone and rostral migratory stream in the hippocampus. The specific distribution of NEIL3 in the central nervous system suggested that NEIL3 plays an important role in neurogenesis. Neural stem/progenitor cells from aged *NEIL3* gene knockout mice showed impaired proliferative capacity and reduced DNA repair activity, indicating that NEIL3 was critical in maintaining hippocampal neurogenesis.[20,21] Our previous studies found that GSRd promoted neurogenesis *in vitro* and *in vivo*[22] and also showed the ability to maintain neural stem cell proliferation in a lead exposure rat model.[23] In this study, we found that GSRd-induced NEIL3 expression in the hippocampus could be one of the mechanisms contributing to neurogenesis promotion, leading to the improvement of survival rates and neurological scores in GSRd-treated MCAO rats. In line with these results, Sejersted *et al*.[9] found that NEIL3 could promote neurogenesis after hypoxia-ischemia. In contrast to NEIL3, NEIL1 is ubiquitously expressed in the brain and may have a role in protecting all types of neuronal cells against oxidative stress.

In conclusion, this study found that the neuroprotective function of GSRd could be partially explained by the upregulation of NEIL1 and NEIL3 expressions. Future studies are needed to determine the potential role of NEILs as a treatment biomarker for ischemic stroke.

Financial support and sponsorship

This study was supported by a grant from the National Natural Science Foundation of China (No. 81371365).

Conflicts of interest

There are no conflicts of interest.

References

1. Mukherjee D, Patil CG. Epidemiology and the global burden of stroke. World Neurosurg 2011;76 6 Suppl:S85-90. doi: 10.1016/j.wneu.2011.07.023.
2. Chen H, Yoshioka H, Kim GS, Jung JE, Okami N, Sakata H, *et al.* Oxidative stress in ischemic brain damage: Mechanisms of cell death and potential molecular targets for neuroprotection. Antioxid Redox Signal 2011;14:1505-17. doi: 10.1089/ars.2010.3576.
3. Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol 2005;25:29-38. doi: 10.1161/01.ATV.0000150649.39934.13.
4. Li P, Hu X, Gan Y, Gao Y, Liang W, Chen J. Mechanistic insight into DNA damage and repair in ischemic stroke: Exploiting the base excision repair pathway as a model of neuroprotection. Antioxid Redox Signal 2011;14:1905-18. doi: 10.1089/ars.2010.3451.
5. Wilson DM 3rd, Bohr VA. The mechanics of base excision repair, and its relationship to aging and disease. DNA Repair (Amst) 2007;6:544-59. doi: 10.1016/j.dnarep.2006.10.017.
6. Ide H, Kotera M. Human DNA glycosylases involved in the repair of oxidatively damaged DNA. Biol Pharm Bull 2004;27:480-5. doi: 10.1248/bpb.27.480.
7. Canugovi C, Yoon JS, Feldman NH, Croteau DL, Mattson MP, Bohr VA. Endonuclease VIII-like 1 (NEIL1) promotes short-term spatial memory retention and protects from ischemic stroke-induced brain dysfunction and death in mice. Proc Natl Acad Sci U S A 2012;109:14948-53. doi: 10.1073/pnas.1204156109.
8. Chakraborty A, Wakamiya M, Venkova-Canova T, Pandita RK, Aguilera-Aguirre L, Sarker AH, *et al.* Neil2-null mice accumulate oxidized DNA bases in the transcriptionally active sequences of the genome and are susceptible to innate inflammation. J Biol Chem 2015;290:24636-48. doi: 10.1074/jbc.M115.658146.

9. Sejersted Y, Hildrestrand GA, Kunke D, Rolseth V, Krokeide SZ, Neurauter CG, *et al.* Endonuclease VIII-like 3 (Neil3) DNA glycosylase promotes neurogenesis induced by hypoxia-ischemia. Proc Natl Acad Sci U S A 2011;108:18802-7. doi: 10.1073/pnas.1106880108.
10. Zhang X, Shi M, Bjørås M, Wang W, Zhang G, Han J, *et al.* Ginsenoside Rd promotes glutamate clearance by up-regulating glial glutamate transporter GLT-1 via PI3K/AKT and ERK1/2 pathways. Front Pharmacol 2013;4:152. doi: 10.3389/fphar.2013.00152.
11. Liu X, Wang L, Wen A, Yang J, Yan Y, Song Y, *et al.* Ginsenoside-Rd improves outcome of acute ischaemic stroke – A randomized, double-blind, placebo-controlled, multicenter trial. Eur J Neurol 2012;19:855-63. doi: 10.1111/j.1468-1331.2011.03634.x.
12. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989;20:84-91. doi: 10.1161/01.STR.20.1.84.
13. Wang W, Esbensen Y, Scheffler K, Eide L. Analysis of mitochondrial DNA and RNA integrity by a real-time qPCR-based method. Methods Mol Biol 2015;1264:97-106. doi: 10.1007/978-1-4939-2257-4_10.
14. Ye R, Kong X, Yang Q, Zhang Y, Han J, Zhao G. Ginsenoside Rd attenuates redox imbalance and improves stroke outcome after focal cerebral ischemia in aged mice. Neuropharmacology 2011;61:815-24. doi: 10.1016/j.neuropharm.2011.05.029.
15. Ye R, Yang Q, Kong X, Han J, Zhang X, Zhang Y, *et al.* Ginsenoside Rd attenuates early oxidative damage and sequential inflammatory response after transient focal ischemia in rats. Neurochem Int 2011;58:391-8. doi: 10.1016/j.neuint.2010.12.015.
16. Chen XC, Zhu YG, Zhu LA, Huang C, Chen Y, Chen LM, *et al.* Ginsenoside Rg1 attenuates dopamine-induced apoptosis in PC12 cells by suppressing oxidative stress. Eur J Pharmacol 2003;473:1-7. doi: 10.1016/S0014-2999(03)01945-9.
17. Wang S, Xing Z, Vosler PS, Yin H, Li W, Zhang F, *et al.* Cellular NAD replenishment confers marked neuroprotection against ischemic cell death: Role of enhanced DNA repair. Stroke 2008;39:2587-95. doi: 10.1161/STROKEAHA.107.509158.
18. He M, Xing S, Yang B, Zhao L, Hua H, Liang Z, *et al.* Ebselen attenuates oxidative DNA damage and enhances its repair activity in the thalamus after focal cortical infarction in hypertensive rats. Brain Res 2007;1181:83-92. doi: 10.1016/j.brainres.2007.08.072.
19. Rolseth, V, Runden-Pran E, Neurauter CG, Yndestad A, Luna L. Base excision repair activities in organotypic hippocampal slice cultures exposed to oxygen and glucose deprivation. DNA Repair (Amst) 2008,7:869-78. doi: 10.1016/j.dnarep.2008.02.009.
20. Reis A, Hermanson O. The DNA glycosylases OGG1 and NEIL3 influence differentiation potential, proliferation, and senescence-associated signs in neural stem cells. Biochem Biophys Res Commun 2012;423:621-6. doi: 10.1016/j.bbrc.2012.04.125.
21. Regnell CE, Hildrestrand GA, Sejersted Y, Medin T, Moldestad O, Rolseth V, *et al.* Hippocampal adult neurogenesis is maintained by Neil3-dependent repair of oxidative DNA lesions in neural progenitor cells. Cell Rep 2012;2:503-10. doi: 10.1016/j.celrep.2012.08.008.
22. Lin T, Liu Y, Shi M, Liu X, Li L, Liu Y, *et al.* Promotive effect of ginsenoside Rd on proliferation of neural stem cells *in vivo* and *in vitro*. J Ethnopharmacol 2012;142:754-61. doi: 10.1016/j.jep.2012.05.057.
23. Wang B, Feng G, Tang C, Wang L, Cheng H, Zhang Y, *et al.* Ginsenoside Rd maintains adult neural stem cell proliferation during lead-impaired neurogenesis. Neurol Sci 2013;34:1181-8. doi: 10.1007/s10072-012-1215-6.