Int J Clin Exp Med 2018;11(11):12326-12332
www.ijcem.com /ISSN:1940-5901/IJCEM0072586

Original Article

Neuroprotective effects of genistein-3'-sodium sulfonate in a rat middle cerebral artery occlusion model: roles of enhancing antioxidant ability and regulating NO/NOS system

Jinhua Xue[1,2,3*], Xiao Li[2,3*], Jiali Xie[2], Ruizhen Liu[2], Cheng Huang[2,3], Zhihua Huang[2,3], Liangdong Li[2,4], Lu Zhang[1]

[1]Laboratory of Functional Proteomics of Guangdong Province, Key Laboratory of Mental Health of The Ministry of Education, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China; [2]Key Laboratory of Prevention and Treatment of Cardiovascular and Cerebrovascular Diseases of Ministry of Education, Gannan Medical University, Ganzhou, China; [3]Institute for Medical Sciences of Pain, Department of Physiology, School of Basic Medical Sciences, Gannan Medical University, Ganzhou, China; [4]The First Affiliated Hospital of Gannan Medical University, Ganzhou, China. [*]Equal contributors.

Received January 11, 2018; Accepted June 20, 2018; Epub November 15, 2018; Published November 30, 2018

Abstract: Genistein-3'-sodium sulfonate (GSS) has better water solubility and stronger anti-peroxidation activity for lipids than genistein, a soy-derived isoflavanoid compound extracted from food sources. A previous study revealed that GSS protects rat cortical neurons from injury induced by focal cerebral ischemia. The present study aimed to investigate whether GSS protects cerebral ischemia-reperfusion injury by enhancing antioxidant ability of the brain. After treatment with GSS, proliferation and LDH levels of hippocampal neurons, injured by serum deprivation, were examined. Following treatment with GSS for 24 hours, brain tissues of rats, subjected to middle cerebral artery occlusion/reperfusion (MCAO/R) or a sham operation, were isolated for detection of SOD, GSH-Px, CAT, T-AOC, tNOS, iNOS, and cNOS activity, as well as MDA and NO content. Results showed that GSS treatment significantly increased neuronal viability and inhibited LDH release in a serum deprivation-induced hippocampal neuron injury model. GSS treatment significantly increased activities of SOD, GSH-Px, CAT, and T-AOC, which were reduced by MCAO/R, and decreased the content of MDA elevated by MCAO/R. Furthermore, GSS treatment significantly improved tNOS and cNOS activity. NO content was reduced by MCAO/R, which also inhibited iNOS activity. In conclusion, GSS-protected hippocampal neurons in an *in vitro* serum deprivation-induced injury model enhanced the antioxidant ability and NO/NOS system of brain tissues in a MCAO/R model. These results suggest that the neuroprotective effects of GSS are dependent upon enhanced antioxidant ability and regulation of the NO/NOS system.

Keywords: Genistein-3'-sodium sulfonate, MCAO/R model, antioxidant ability, nitric oxide synthase, nitric oxide

Introduction

Strokes, including hemorrhagic strokes, ischemic strokes, and their complications, are a leading cause of death [1, 2]. Despite reduced morbidity in some developed countries, mortality in stroke patients remains high worldwide [1]. Ischemic strokes account for 80% of all stroke patients [3]. Because incidence of ischemic strokes increases with age, it is expected that this problem will become increasingly urgent in an aging society. Treatment for this disorder continues to be limited, only palliative in nature. At present, drugs for ischemic stroke are limited to antiplatelet drugs, anticoagulants, thrombolytic agents, vasodilators, and brain protectants. However, anti-cerebral ischemic drugs, which have significant curative effects, limited side effects, and multiple targets, are still very limited in number. Currently, there is no effective pharmacotherapy for this illness. Therefore, screening for effective medicines to improve impairments caused by ischemic stroke is an urgent mission in neuroscience research, having significant economic benefit and social significance.

Traditional Chinese Medicine exerts pharmacological effects through a multi-component and multi-target approach, including monomer components [4]. Therefore, it should be feasible to screen effective monomer components from Traditional Chinese Medicine to improve impairments caused by ischemic strokes. Genistein (4',5,7-trihydroxyisoflavone) is a soy derived isoflavanoid compound extracted from food sources, such as tofu, fava beans, soybeans, kudzu, and lupin [5, 6]. Genistein has preventive and therapeutic effects on cerebral ischemia and other models of brain injury by attenuating oxidative stress, promoting growth factor signaling, and suppressing inflammation [7]. Genistein also has vasodilatory effects via the eNOS pathway [8]. Genistein-3'-sodium sulfonate ($C_{15}H_{10}O_8SNa$), a relatively more water-soluble compound, is a structural modification of genistein [9]. A previous study revealed that GSS protected rat cortical neurons from injuries induced by focal cerebral ischemia in both *in vitro* and *in vivo* models [9]. In addition, GSS has stronger anti-peroxidation activity for lipids than genistein [10]. Based on these results, it was hypothesized that the protective effects of GSS on cerebral ischemia-reperfusion injury might depend on enhancement of antioxidant ability.

The present study investigated whether GSS can enhance the antioxidant ability of brain tissue in a middle cerebral artery occlusion (MCAO)/reperfusion (MCAO/R) model, a reliable method for studying reversible regional ischemia [11]. In addition, this study investigated whether GSS can enhance the antioxidant ability of hippocampal neurons following serum deprivation in an *in vitro* model of ischemic cell injury. Results indicated that GSS can, in fact, enhance antioxidant ability in both *in vivo* and *in vitro* ischemic injury models. These results provide additional theoretical basis for understanding the protective effects of GSS on cerebral ischemia-reperfusion injury via enhancement of antioxidant ability.

Materials and methods

Animals

Specific pathogen-free (SPF) grade male Sprague-Dawley (SD) rats (250-280 g) and SPF grade pregnant female SD rats were purchased from Hunan Slack Jingda Experimental Animal Co., Ltd (License number: SCXK [Xiang] 2011-0003, Certification No.: 43004700003244; Changsha, Hunan, China).

Isolation and culture of primary hippocampal neurons

Hippocampal neurons were isolated from specific pathogen-free grade fetal SD rats and cultured, as described previously [12]. Briefly, pregnant SD rats, at approximately 18 days post-fertilization, were euthanized by cervical dislocation. Following disinfection of the abdomen with 75% ethanol, the uterus was opened and the fetal rat was removed using autoclaved sterile forceps. The entire brain of the fetal rat was carefully removed with forceps. Afterward, the meninges and blood vessels were removed. The hippocampus was isolated, gently lifted with sterile tissue forceps, and transferred to a small tissue culture dish, then washed with HBSS. The hippocampus was cut into small pieces with a diameter of 1 mm and digested by 0.25% Trypsin solution at 37°C for 30 minutes. The tissue pellet was washed with 5 mL of HBSS at 37°C for 5 minutes. This was repeated three times. The final wash was removed from the tissue pellet and replaced with 2 mL of fresh Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA). Using a standard sterile 9-inch Pasteur pipette, the tissue was gently triturated seven times. After allowing the solution to precipitate for three minutes, dissociated cells of the supernatant were counted using a hemocytometer, diluted to 1×10^5 cells/well, and then cultured in a 37°C, 5% CO_2 incubator for 4 hours. DMEM was carefully removed and neurobasal media (Gibco) containing 2% (v/v) B27 supplement (Gibco) and 1% (v/v) Glutamax (Gibco) was added to the plates and the neurons were cultured in a 37°C, 5% CO_2 incubator for three days. After three days, half of the media was removed from the cells and replaced with an equal volume of neurobasal media containing 2% (v/v) B27 supplement, 1% (v/v) Glutamax, and 1% (v/v) cytarabine (Gibco). The neurons were again cultured in a 37°C 5% CO_2 incubator for three days. Cultured neurons continued to be fed every three days by removing half of the old media and replacing it with an equal volume of fresh neurobasal feeding media. Neurons were cultured for eight days for further experiments.

Serum deprivation-induced hippocampal neuron injury model and GSS treatment

After being cultured for eight days, the neurons were divided into six groups: control group and 0, 10, 30, 100, and 300 μM GSS treatment group. Briefly, neurons were pretreated with

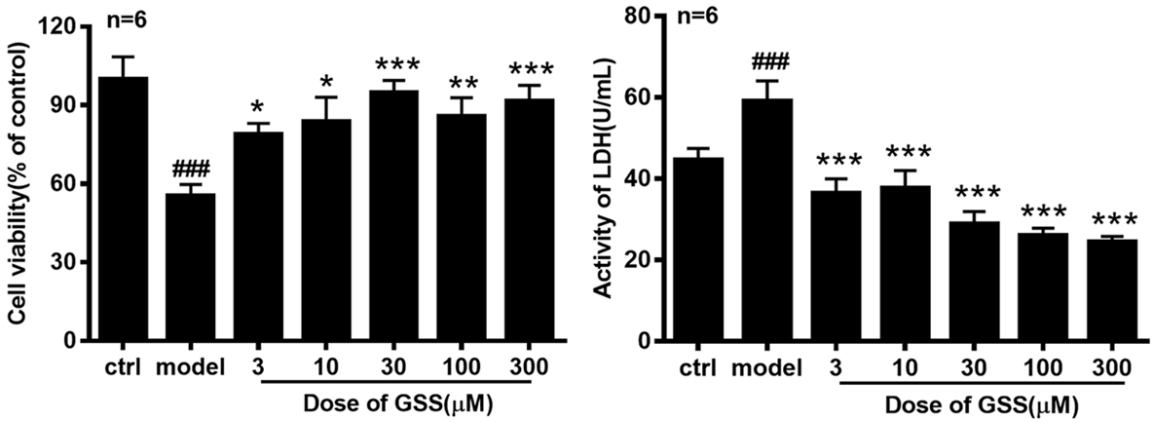

Figure 1. Hippocampal neuronal viability and LDH activity was determined after GSS treatment for 24 hour, *in vitro*. Control group (ctrl) consisted of neuronal cells cultured with 10% FBS. Other groups consisted of neuronal cells cultured in the absence of 10% FBS and treated with (0, 3, 10, 30, 100, and 300 μM) GSS. Results are expressed as mean ± SD. ###P < 0.001 vs. control group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the serum deprivation-treated group.

GSS at specified concentrations (0, 3, 10, 30, 100, and 300 μM) for 30 minutes. Neurobasal media was removed and DMEM without fetal bovine serum (FBS, Gibco) was added. To induce the neuronal injury model, neurons were then cultured in a 37°C, 5% CO_2 incubator for 24 hours. In the control group, neurons were cultured in DMEM with 10% FBS. Each group included six replicates.

Cell viability assay

Cell viability was determined using 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide (MTT) reduction assay (Sigma, USA). After treatment for 24 hours, 20 μL MTT (5 mg/mL) was added to cells in each group and incubated for an additional 4 hours at 37°C. Absorbance was recorded at 570 nm using a microplate reader (Varioskan LUX, Thermo Scientific, Vantaa, Finland).

LDH release assay

After treatment for 24 hours, cell culture supernatant was collected. Measurement of LDH activity in the supernatant was performed according to protocol provided by the manufacturer (Nanjing Jiancheng, Nanjing, China).

Establishment of MCAO reperfusion model and treatment with GSS

Adult male SD rats were housed in climate-controlled rooms on a 12 hour light-dark cycle with free access to food and water. All experimental procedures conformed to guidelines of the Animal Care and Use Committee of Gannan Medical University. Effort was made to minimize discomfort to the animals. Forty adult rats were divided randomly into 5 groups (n=8): sham group, model group (0 mg/kg GSS treatment), 0.5 mg/kg GSS treatment group, 1 mg/kg GSS treatment group, and the 2 mg/kg GSS treatment group. This rat middle cerebral artery occlusion (MCAO) model has been described in a previous study [11]. Rats were anesthetized with 350 mg/kg chloral hydrate by intraperitoneal injection. Next, a 4-cm-long nylon filament (diameter 0.26 mm) was inserted into the middle cerebral artery, inducing ischemia. Ten minutes after ischemia was initiated, rats were treated with GSS at specified concentrations (0, 0.5, 1.0, and 2.0 mg/kg) by sublingual intravenous injection. After 2 hours, the nylon filament was carefully removed to allow blood to return to the ischemic artery, then sutured to establish reperfusion. Sham-operated rats were subjected to the same surgical procedure as rats in the MCAO group, except for occlusion of the middle cerebral artery. They were treated with an equivalent volume of normal saline.

Enzyme activity assay

Rats were sacrificed 24 hours after reperfusion and ischemic-side forebrain was collected and homogenized with cold normal saline. The tissue homogenate was centrifuged at 3500 rpm

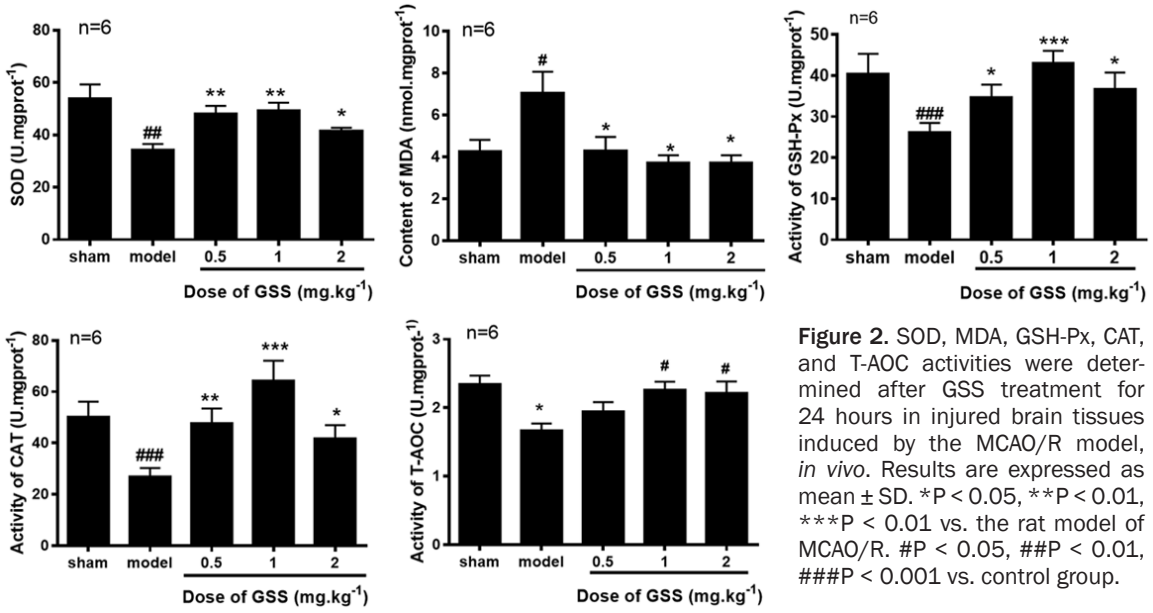

Figure 2. SOD, MDA, GSH-Px, CAT, and T-AOC activities were determined after GSS treatment for 24 hours in injured brain tissues induced by the MCAO/R model, *in vivo*. Results are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.01 vs. the rat model of MCAO/R. #P < 0.05, ##P < 0.01, ###P < 0.001 vs. control group.

for 10 minutes at 4°C and supernatant was collected for subsequent assays. Antioxidant status of the brain was assessed by the activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), total antioxidant capacity (T-AOC), total nitric oxide synthase (tNOS), inducible nitric oxide synthase (iNOS), constitutive nitric oxide synthase (cNOS), nitric oxide (NO), and malondialdehyde (MDA), according to manufacturer protocol (Nanjing Jiancheng).

Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) v19.0 software (SPSS Inc, Chicago, IL, USA). All data are expressed as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) followed by post-hoc tests of Student-Newman-Keuls (SNK) for multiple pairwise comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

GSS ameliorates injury in serum deprivation-induced hippocampal neuron injury model

After EAS treatment, proliferation of hippocampal neurons and LDH levels were measured. Results are shown in **Figure 1**. Serum deprivation significantly reduced neuronal viability and increased LDH activity in neurons (P < 0.05), while GSS treatment significantly increased neuronal viability and inhibited LDH activity (P < 0.05) in the serum deprivation-induced hippocampal neuron injury model.

GSS improves antioxidant capacity in rat model of MCAO reperfusion

As shown in **Figure 2**, SOD, GSH-Px, CAT, and T-AOC activities in brain tissues from the rat MCAO/R model were significantly lower than those in the sham group (P < 0.05). MDA content in brain tissues from the rat MCAO/R model was significantly higher than the sham group (P < 0.05). Compared with the MCAO/R model group, GSS treatment significantly increased SOD, GSH-Px, CAT, and T-AOC activities reduced by MCAO/R (P < 0.05) and decreased MDA content enhanced by MCAO/R (P < 0.05).

GSS improves NO metabolism in rat model of MCAO reperfusion

As shown in **Figure 3**, tNOS activity, cNOS activity, and NO levels in brain tissues from the rat MCAO/R model were significantly lower, while iNOS activity was significantly higher, than the sham group (P < 0.05). Compared with the MCAO/R model group, GSS treatment significantly increased tNOS activity, cNOS activity, and NO content previously reduced by MCAO/R (P < 0.05), while decreasing iNOS activity that was increased by MCAO/R (P < 0.05).

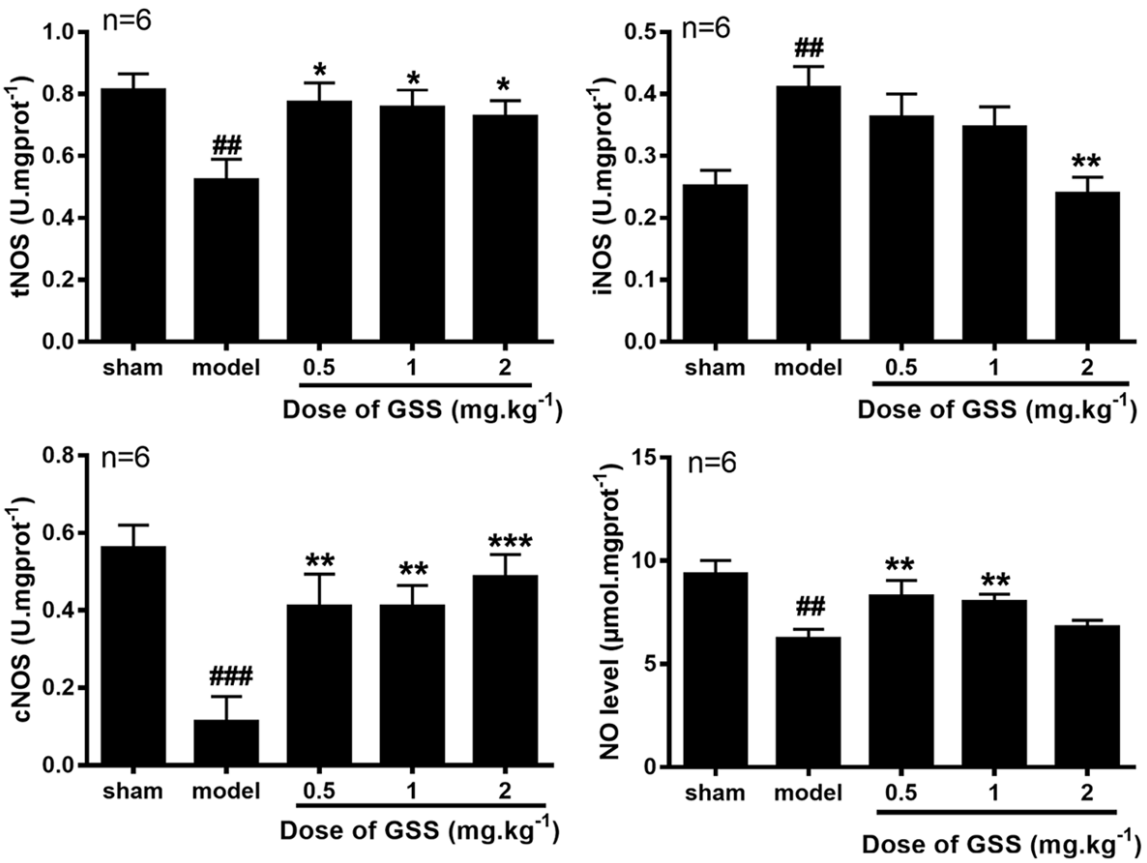

Figure 3. tNOS, iNOS, cNOS, and NO levels in brain tissues were determined after GSS treatment for 24 hours in injured brain tissues induced by the MCAO/R model, *in vivo*. Results are expressed as mean ± SD. *P < 0.05, *P < 0.05, **P < 0.01, ***P < 0.001 vs. the rat model of MCAO/R. ##P < 0.01, ###P < 0.001 vs. control group.

Discussion

A previous study indicated that GSS has stronger anti-peroxidation activity for lipids than genistein [10]. However, it is not clear whether GSS can enhance the antioxidant ability of the brain and reduce injury caused by cerebral ischemia-reperfusion. The present study demonstrates that GSS treatment can enhance activities of SOD, GSH-Px, CAT, and total antioxidant capacity while also decreasing MDA levels in MCAO/R-injured brains. In addition, it was found that GSS treatment can enhance the content of tNOS, cNOS, and NO while decreasing iNOS levels. Furthermore, this study found decreased LDH activity in the culture medium and increased cell viability of hippocampal neurons. These results indicate that GSS can enhance the antioxidant ability of the brain in *in vivo* and *in vitro* ischemic injury models.

Oxidative stress, referring to a relative surplus of reactive oxygen species (ROS) caused by excessive ROS generation and/or impaired ROS degradation, plays an important role in the pathological mechanisms of ischemic stroke [13]. In the pathological process of ischemic stroke followed by reperfusion, excessive amounts of ROS cause lipid, protein, and nucleic acid peroxidation as well as cell membrane damage, eventually leading to neuron damage [14]. Thus, it is important for the brain's normal metabolism to maintain a balance between ROS production and degradation. Excessive ROS can be scavenged by antioxidant defense systems, such as SODs, CATs, and GSH-Px [15]. This present study found that GSS treatment can enhance activities of SOD, GSH-Px, and CAT in injured brains induced by MCAO/R. These results revealed that GSS treatment enhanced antioxidant ability in the context of pathological mechanisms following cerebral ischemia-reperfusion injury. MDA, one of the final products of peroxidation of unsaturated fatty acids in phospholipids, is responsible for cell membrane damage [16]. It was found that

GSS treatment can decrease MDA levels in injured brains induced by MCAO/R and increase LDH activity in a serum deprivation-induced hippocampal neuron injury model, further indicating an antioxidant role of GSS in cerebral ischemia-reperfusion injury.

NO, which has emerged as a neural messenger, plays an important role in ischemia [8, 17]. NO can be synthesized by NOS from L-arginine. There are three known isoforms in mammals. Two are constitutive (cNOS) while the other is inducible (iNOS) [18]. cNOS includes neuronal constitutive NOS (nNOS) and endothelial constitutive NOS (eNOS). Several studies have strongly supported the hypothesis that nNOS activation is detrimental to the ischemic brain [19]. Evidence, particularly from studies with NOS inhibitors, suggests that post-ischemic NO generated by eNOS may indeed be protective [20]. However, iNOS expression is usually excessively induced in an oxidative environment. Thus, high levels of NO generated by iNOS have the potential to react with superoxide, leading to peroxynitrite formation and cell toxicity [19-21]. Therefore, excessive expression of iNOS is harmful to brain tissue [19]. Evidence has also shown that NO pathways play a vital role in the neuroprotective mechanism of preconditioning and postconditioning, but these effects were abolished by pretreatment with L-NAME, a nonselective NOS inhibitor [22, 23]. The present study found that GSS treatment can enhance the content of cNOS and NO and decrease iNOS levels in injured brains induced by MCAO/R. nNOS activity increases 10 minutes after focal ischemia and returns to normal after 60 minutes [24], thus, the main constituent of cNOS may be eNOS. On one hand, GSS treatment can increase the content of NO. On the other hand, GSS treatment enhances the activity of eNOS, which is protective and decreases iNOS levels that are harmful to tissues in injured brains induced by MCAO/R. These results further demonstrate that GSS plays a protective role in cerebral ischemia-reperfusion injury.

In conclusion, GSS enhanced the antioxidant ability of brain tissues in a MCAO/R model and the antioxidant ability of hippocampal neurons in an *in vitro* serum deprivation-induced injury model. In addition, GSS also enhanced the content of NO and eNOS, which are protective, and decreased iNOS levels, which are harmful to brain tissues in injured brains induced by MCAO/R. These results suggest that neuroprotective effects of GSS in a rat MCAO/R model are dependent upon enhanced antioxidant ability and regulation of the NOS system. Results of this present study give the theoretical basis to further prove the possibility of GSS as an effective drug improving impairment caused by ischemic strokes.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (31360250, 81760654), the Jiangxi Natural Science Foundation (20122BAB205037), and the Bureau of Education (GJJ12554) of Jiangxi Province.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Liangdong Li, The First Affiliated Hospital of Gannan Medical University, No. 23, Qingnian Road, Ganzhou 341000, Jiangxi, China. Tel: +86 20 61648726; E-mail: gnyxylld@163.com; Dr. Lu Zhang, Laboratory of Functional Proteomics of Guangdong Province, Key Laboratory of Mental Health of The Ministry of Education, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China. Tel: +86 20 61648726; E-mail: zlulu70@126.com

References

[1] Zhang S, He WB and Chen NH. Causes of death among persons who survive an acute ischemic stroke. Curr Neurol Neurosci Rep 2014; 14: 467.

[2] Chan PH. Handbook of neurochemistry and molecular neurobiology. Springer Verlag; 2006.

[3] Wang Y, Shen J, Wang XM, Fu DL, Chen CY, Lu LY, Lu L, Xie CL, Fang JQ and Zheng GQ. Scalp acupuncture for acute ischemic stroke: a meta-analysis of randomized controlled trials. Evid Based Complement Alternat Med 2012; 2012: 480950.

[4] Du G, Wang Y, Zhang R, Tan C, He X, Hu J, Zhang L, Chen R and Qin H. Multi-target and multi-component pattern,a superficial understanding of the action mechanism of Traditional Chinese Medicine. World Science & Technology 2009; 4: 480-484.

[5] Ganai AA and Farooqi H. Bioactivity of genistein: a review of in vitro and in vivo studies. Biomed Pharmacother 2015; 76: 30-38.

[6] Spagnuolo C, Russo GL, Orhan IE, Habtemariam S, Daglia M, Sureda A, Nabavi SF, Devi KP, Loizzo MR, Tundis R and Nabavi SM. Genistein and cancer: current status, challenges, and future directions. Adv Nutr 2015; 6: 408-419.
[7] Schreihofer DA and Oppong-Gyebi A. Genistein: mechanisms of action for a pleiotropic neuroprotective agent in stroke. Nutr Neurosci 2017; 24: 1-17.
[8] Liu K, Yan M, Zheng X and Yang Y. The dynamic detection of NO during the ischemic postconditioning against global cerebral ischemia/reperfusion injury. Nitric Oxide 2014; 38: 17-25.
[9] Li L, Xue J, Liu R, Li X, Lai L, Xie J, Huang Z and Huang C. Neuroprotective effects of genistein-3'-sodium sulfonate on focal cerebral ischemia in rats. Neurosci Lett 2017; 646: 43-48.
[10] Suo Z, Zhang Z and Zheng J. Preparation of natrium sulfogenisteinates and their anti peroxidation activity for lipids (In Chinese). Chinese Journal of Applied Chemistry 2005; 22: 1083-1086.
[11] Longa EZ, Weinstein PR, Carlson S and Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989; 20: 84-91.
[12] Seibenhener ML and Wooten MW. Isolation and culture of hippocampal neurons from prenatal mice. J Vis Exp 2012.
[13] Toledo-Pereyra LH, Lopez-Neblina F and Toledo AH. Reactive oxygen species and molecular biology of ischemia/reperfusion. Ann Transplant 2004; 9: 81-83.
[14] Luca M, Luca A and Calandra C. The role of oxidative damage in the pathogenesis and progression of alzheimer's disease and vascular dementia. Oxid Med Cell Longev 2015; 2015: 504678.
[15] Noctor G and Foyer CH. Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 1998; 49: 249-279.
[16] Halliwell B and Gutteridge JM. Free radicals in biology and medicine. Oxford, UK: Oxford University Press; 1989.
[17] Liu H, Li J, Zhao F, Wang H, Qu Y and Mu D. Nitric oxide synthase in hypoxic or ischemic brain injury. Rev Neurosci 2015; 26: 105-117.
[18] Stuehr DJ. Mammalian nitric oxide synthases. Biochim Biophys Acta 1999; 1411: 217-230.
[19] Moro MA, Cardenas A, Hurtado O, Leza JC and Lizasoain I. Role of nitric oxide after brain ischaemia. Cell Calcium 2004; 36: 265-275.
[20] Keynes RG and Garthwaite J. Nitric oxide and its role in ischaemic brain injury. Curr Mol Med 2004; 4: 179-191.
[21] Gulati P and Singh N. Tadalafil enhances the neuroprotective effects of ischemic postconditioning in mice, probably in a nitric oxide associated manner. Can J Physiol Pharmacol 2014; 92: 418-426.
[22] Li Y, Liu K, Kang ZM, Sun XJ, Liu WW and Mao YF. Helium preconditioning protects against neonatal hypoxia-ischemia via nitric oxide mediated up-regulation of antioxidases in a rat model. Behav Brain Res 2016; 300: 31-37.
[23] Gulati P and Singh N. Pharmacological evidence for connection of nitric oxide-mediated pathways in neuroprotective mechanism of ischemic postconditioning in mice. J Pharm Bioallied Sci 2014; 6: 233-240.
[24] Kader A, Frazzini VI, Solomon RA and Trifiletti RR. Nitric oxide production during focal cerebral ischemia in rats. Stroke 1993; 24: 1709-1716.