Shenfu injection improves cognitive dysfunction in vascular dementia mouse model via the NOS/NO pathway

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Abstract. Obsjective: To investigate the treatment effects and underline mechanism of Shenfu injection on cognitive dysfunction in vascular dementia(VD) model mice. Methods: Male mice were randomly divided into a Sham, VD, SFI, L-Arg, and L-NAME group. VD mouse was made by repeated ischemia and perfusion by clipping the bilateral common carotid arteries and simultaneously injecting with sodium nitroprusside intraperitoneally. Mouse was subjected to corresponding pharmacological intervention for 21 days while mouse in sham and VD groups was administered saline. Morris water maze were employed to assess learning and memory ability, and HE and Nissl staining were used to analyze pathological changes. Serum NO level,ROS, MDA, and GSH in brain tissue were assessed by biochemical methods. iNOS, nNOS, and eNOS protein expressions were detected by western blotting. Results: Compared with the VD group, the learning and memory abilities of mice in SFI and L-NAME groups were improved, pathological damage of the hippocampal CA1 region of mice in these groups was significantly improved, NO, ROS, and MDA contents were decreased, GSH level and eNOS protein expression were increased, and iNOS and nNOS protein expression were significantly decreased($P<0.01$, $P<0.05$). However, L-Arg treated mice showed no significant improvements in these indexes when compared to the VD group. Conclusion: SFI may improve the cognitive dysfunction in vascular dementia mouse model via the NOS/NO pathway

Keywords: Shenfu injection; vascular dementia; nitric oxide; nitric oxide synthase; cognitive dysfunction.

1. Introduction

Vascular dementia(VD) is a syndrome characterized by deterioration of memory deterioration and progressive cognitive dysfunction caused by ischemic or hemorrhagic cerebrovascular disease,as well as cardiovascular and circulatory system diseases[1]. VD is second most common form of dementia after Alzheimer's disease(AD)accounting for 15% to 20% of dementia cases.The incidence of the VD has increased with aging cerebrovascular diseases[2].According to the World Health Organization, the population with dementia will double,reaching over 100 million by 2050. However, the underlying pathogenesis of VD remains unclear,and there is a lack of systematic treatment options. Traditional Chinese Medicine has become a research hotspot in exploring new treatment schemes for VD due to its characteristics of multi-components, multi-targets and multi-pathways.Shenfu injection(SFI) which contains ginsenosides from red ginseng and aconite alkaloids from aconite,is widely used clinically for treating coronary heart disease, congestive heart failure, myocardial ischemic reperfusion and other cardiovascular diseases[3]. As the study progressed, the role of SFI in improving neurological diseases was gradually demonstrated[4]. SFI can ameliorate cerebral ischemia and reperfusion injury by alleviating inflammation, nerve cell apoptosis and neurological damage[5-6]. Ohno et al.[7] found that a significant reduction in cognitive dysfunction 24 hours after injecting nitric oxide inhibitor (L-NAME) in whole-ischemic

rats,suggesting that L-NAME protects against neuronal damage in the CA1 region of the hippocampus. Nitric oxide (NO) plays a crucial modulatory role in cerebrovascular injury and neuroinflammation. However,excessive NO can cause neurotoxic damage to neurons resulting to impaired cognition[8]. In the cerebral ischemia-reperfusion injury, NO can influence neuronal survival and integrity of BBB, indicating its close association with this condition. Interventions targeting content of NO and related mechanisms of action may be potential treatments for cerebral ischemia-reperfusion[9]. Wu et al.[4] discovered that the application of SFI after cerebral ischemia and reperfusion significantly reduced the levels of IL-6 and TNF-a by regulating the expression of NOS while decreasing the production of neurotoxic NO.Therefore, in this study, we explored whether SFI could improve the learning and cognitive functions of VD mice through the NOS/NO pathway by evaluating its effect of SFI on VD mice.

2. Materials and Methods

2.1 Animals

Male KM mice were provided by the Experimental Animal Science and Technology Center of Jiangxi University of Traditional Chinese Medicine[SCXK (Gan) 2018-0003].A total of 50 KM mice, weight 20 to 22 g, were kept at SPF environment with barrier at Nanchang University [SYXK (Gan) 2021-0004]. The study was approved by the Experimental Animal Ethics Committee of Nanchang University ((2020) No (19)),and given humane care according to the 3R principle of experimental animals.

2.2 Reagents

Shenfu injection(Sanjiu Pharmaceutical Co., Ltd,Product Batch No.:200807 AK 02);sodium nitroprusside (Solarbio,Item No.: S9560);nitric oxide synthase inhibitor (Item No.: S0006); and nitric oxide precursor (Item No.: S0012) were purchased from Biyuntian Biotechnology Co.,LTD.ROS(Item No.:E004-1-1); MDA test kit(Item No. :A003-1-2); and GSH determination kit (Item No.: A006-2-1) were obtained from Nanjing Jiancheng Bioengineering Institute.Nye staining solution(Solarbio, Item No.:G1430); RIPA Lysate(Beijing General Gene Technology Co.,Ltd.,Item No.:C1053);protein Marker (Shanghai Yisheng Biotechnology Co., Ltd.,No.: 20350 ES76);GAPDH Rat resistance(RuiYing, No.: RLM3029);rabbit anti-nNOS (Cellsignaling, No.: 4234); rabbit anti-eNOS (RuiYing, No.: RLM3164); rabbit anti-iNOS (Boster, No.: BA0362).

2.3 Experimental groups and protocol

After 1 week of adaptive rearing,a total of 50 mice were used for the establishment of the vascular dementia (VD) mouse model. Among them, forty-five mice were randomly selected and anesthetized with 3% barbiturate sodium at a dose of 40mg/kg. The VD model was induced by repeated clipping and reperfusion of bilateral common carotid arteries, as well as intraperitoneal injection of sodium nitroprusside[10-11]. Following anesthesia, the awake state of the mice were assessed using the the Longa scoring criteria:0 point: no symptoms;1 point: internal flexion of the contralateral forelimb during tail lifting;2points: rotation to the opposite side when crawling;3 points: dumping to the opposite side when standing or crawling; 4 points: no autonomous activity with consciousness disorder.Mice with a score equal to or above one were considered successfully modeled and those with motor ability scores ranging from one to three were selected for further experiments. These successfully modeled mice were then randomly divided into 4 groups: VD model group, L-Arg group(25 mg/kg), L-NAME group(10 mg/kg)and SFI group(10 mL/kg)[12-14],each consisting of nine mice.Additionally, nine mice were selected as the Sham operation group, resulting in a total of five groups.On the day following modeling inductioneach,each group received their respective measured doses:SFI group was injected shenfu injection via the tail vein while L-Arg group and L-NAME group were intraperitoneally injected with arginine and nitric oxide inhibitor respectively,Both sham operation and VD model groups

were injected the same amount of physiological saline in the same way. The drugs were administered consecutively for a period lasting twenty-one days.

2.4 Behavioral experiments

The learning and memory abilities of mice were evaluated by the Morris water maze test following a 3-week drug administration period, which was followed by continuous testing for 6 consecutive days. The water maze test was conducted daily at a fixed time.Mice were put to swim four times in four quadrantsin the water maze and the target quadrant platform was placed 1 cm below the water surface for the first 5 days of the experiment.The time when the mice found the platform was recorded as the learning time.Removing the platform on Day 6,the resident time of mice in the target quadrant and the number of times crossing the target quadrant platform were recorded and denoted as memory time.

2.5 Preparations of tissue samples

After the behavioral trial, food and water were withheld on the same day, and materials were taken on the next day.Eye blood samples were obtained from mice treated with 3% barbiturate sodium at a dosage of 40 mg/kg to prepare serum.Six mice were randomly selected form each group for sacrifice and their brain tissue was dissected to collect the hippocampus and cerebral cortex separately. The brain tissue was then transferred to an 80℃ refrigerator for preservation. The remaining mice underwent anesthesia, and their chest cavities were opened to expose the heart. A needle was inserted from the left ventricular apex into the ascending aorta. Subsequently, saline (25 mL) was administered through cutting the right atrial appendage until clear outflow was observed; this was followed by perfusion with 100 mL of paraformaldehyde buffer solution (4%). Perfusion was immediately stopped when liver color turned white and mouse limbs became stiff and tonic. After craniotomy, the whole brain was removed and placed in paraformaldehyde solution for fixation prior to tissue staining.

2.6 Histopathological analysis

(1)HE staining: After routine dewaxing of sections, hematoxylin was applied for 5 minutes, differentiated using 1% hydrochloric acid, eosin restained for 2 minutes, subjected to gradient dehydration, sealed with glycerol gelatin, and promptly photographed to observe the morphological changes of neuronal cells in the CA1 hippocampus of the brain.

(2)Nissle staining: After routine dewaxing, the tar violet oil dye was immersed in a temperature-controlled box at 56℃ for 1 hour. Subsequently, it was rinsed three times with ddH2O,placed in Nissl, and differentiated for 2 mins.The tissue underwent gradient dehydration to achieve transparency before being sealed with neutral gum.,Finally,photographed as soon as possible to observe the morphology of brain tissue.

2.7 Measurement of NO Formation

NO formation was estimated by measuring the stable endproduct of NO in aqueous solutions, nitrite (NO2-), by using Griess reagent (0.1% N-(1-naphthyl)- ethylenediamine and 1% sulfanilamide in H3PO4) . The nitrite concentration was determined using a curve calibrated on sodium nitrite standard.

2.8 Immunoblot analysis

Total protein was extracted, prepared and pre-cooled with a 1;100 proportional protease inhibitor and RIPA lysate. Tissue samples weighing 50 to 100 mg were weighed into 1.5 mL centrifuge tubes,followed by the addition of 1mL of lysate.Homogenated was performed until no visible tissue mass .was observed The mixture was then incubated on ice for 30 minutes and shaken every 10 minutes on a vortex mixing instrument for 15 seconds. Subsequently, it was centrifuged at 12,000 r/min and 4℃ for 10 minutes, after which the supernatant was transferred to a new centrifuge tube

(1.5 mL). The protein amount of each group was measured and recorded,ensuring equal amounts of total protein were calculated for subsequent gel electrophoresis, electroporation and sealing closed.PVDF membrane was incubated with the rabbit anti-rat nNOS antibody (1:1000), iNOS antibody (1:1000), eNOS antibody (1:2000) and GAPDH (1:5000) at 4℃ overnight.After exposure,incubated corresponding secondary antibody was photographed and analysed using Image J Software to determine the gray values in order to calculate the levels of protein expression.

2.9 Detection of oxidative stress

The hippocampus was accurately weighed.Subsequently, nine times the volume of homogenized medium was added in a weight-to-volume ratio of 1:9 (g:mL) and mechanically homogenized under an ice bath.After homogenization, the sample was centrifuged for 10 minutes, and the resulting supernatant was measured. Following the instructions provided with the kit (ROS, MDA, GSH), the experiment was conducted in 96-well plates and incubated at 37℃ for 10 minutes. The absorbance values at a wavelength of 510 nm were then measured for each well.

2.10 Statistical analysis

Values were expressed as mean±SD, analyzed using one-way analysis of variance(ANOVA) and Student's t-tests in the program SPSS19.0(USA). P-values of less than 0.05 were considered to be significant.

3. Results

3.1 Effect of SFI on the behavior of VD mice

The results of the water maze experimental localization navigation test demonstrated that compared with the sham operation group, the latency time of localization navigation was significantly prolonged in the VD group($P<0.01$).In contrast, there were no significant alterations in the latency of localization navigation between the L-Arg group and VD group. However,both SFI and L-NAME groups exhibited a shortened latency of localization navigation compared to the AD group($P<0.05$).(Graph 1A,1B).Morris water maze spatial exploration test revealed a significant reduction in the residence time and number of platform visits in the target quadrant (fourth quadrant) for the VD group compared to the sham operation group($P<0.05$).However, there was no significant increase observed in the residence time and number of platform visits in the target quadrant for the L-Arginine group compared with the VD group. In contrast, both SFI and L-NAME groups exhibited a marked increase in these parameters ($P < 0.05$). These findings demonstrate that SFI and L-NAME effectively enhance learning and memory function in VD mice.(Graph 1C,1D).

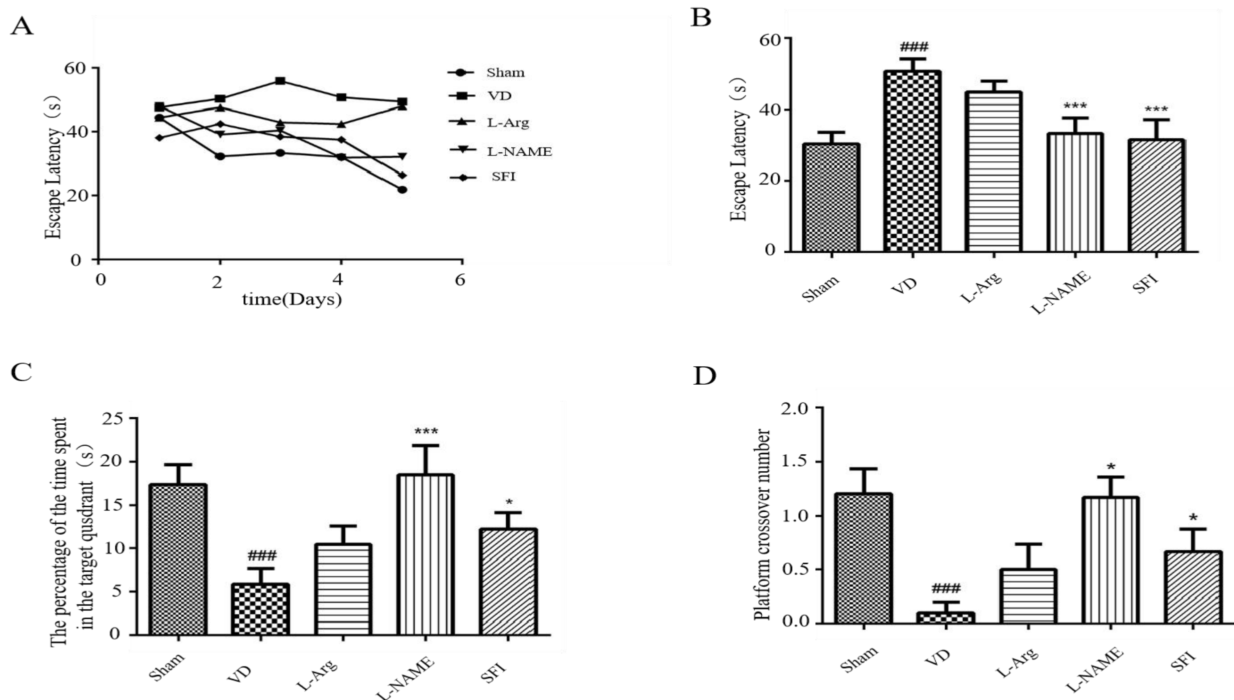

Figure 1 Morris water maze test in Sham, VD, L-Arg, L-NAME and SFI groups. A and B, Latent period. C, The percentage of the time spent in the target quadrant. D, Number of crossover the target platform. Compared with the sham group, ###$P<0.01$. Compared with the VD model group, ***$P<0.01$, *$P<0.05$.

3.2 Effect of SFI on the pathological histology of the CA1 region in the hippocampus of VD mice

3.2.1 H&E staining

Compared with the sham operation group, VD group exhibited structural alterations in the hippocampus,including sparse cells,thinning of cell layers, even loss of cell layers, deformation of neuronal cell bodies, and increased staining and sequestration of cells. In comparison to the VD group, the L-Arg group displayed more orderly aligned hippocampal neurons with similar structures and slightly modified cell layers.The SFI group and L-NAME group showed clear hippocampal structure with thickened cell layers, deep staining and fixation of some neuronal nuclei, as well as polygonal changes in cell bodies; however, pathological damage was still evident when compared to the VD group(Figure 2).

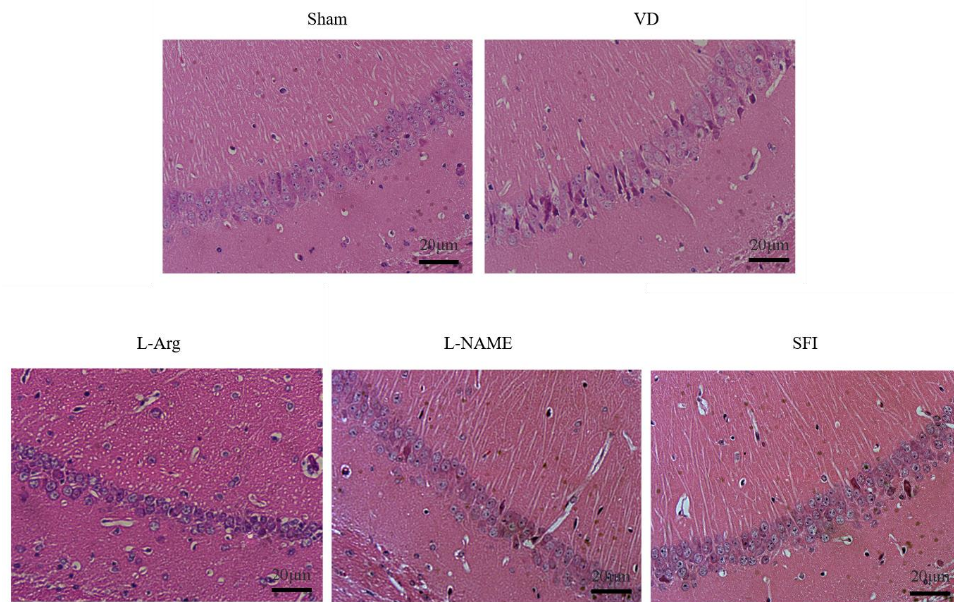

Figure 2 H&E staining of pathological tissues in CA1 region of mouse hippocampus in Sham, VD, L-Arg, L-NAME and SFI groups.

3.2.2 Nissle staining

Compared with the sham operation group, hippocampal CA1 area of mice in the VD group exhibited a reduction in neuronal count, cellular contraction, deep cytoplasm staining, decreased number of positive cells, and an absence of distinct cell morphology. In contrast to the VD group, both SFI and L-NAME groups demonstrated an increase in neuronal cells with predominantly clear and normal morphology. Additionally, these groups displayed reduced deep cytoplasm staining and enhanced neliabase recovery. However, no significant changes were observed in cell morphology or Nissl staining between the L-Arg group and the VD group (Figure 3)

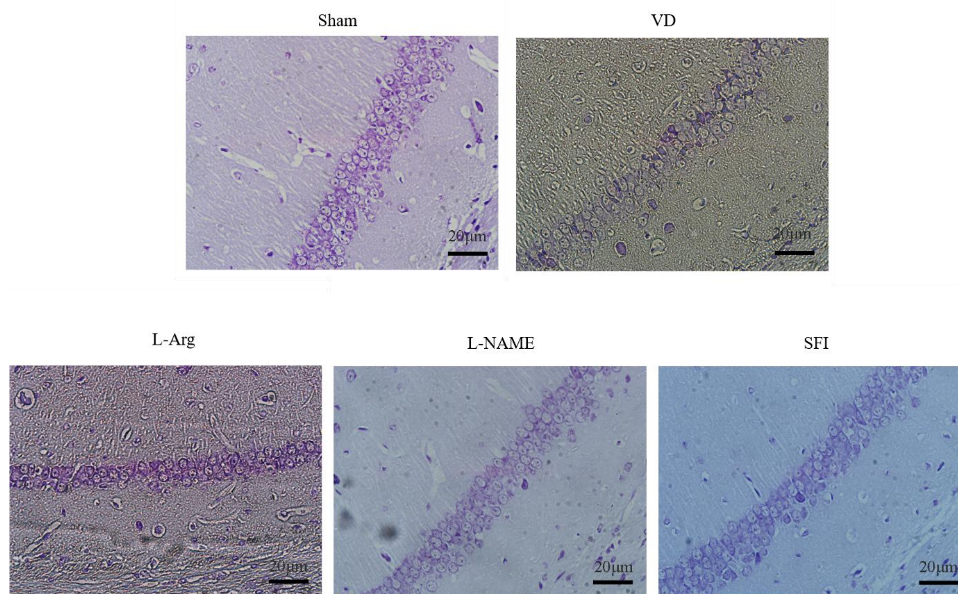

Figure 3 Nissle staining of pathological tissues in CA1 region of mouse hippocampus in Sham, VD, L-Arg, L-NAME and SFI groups

3.3 Effect of SFI on serum NO content in VD mice

The serum nitric oxide (NO) content was significantly higher in the VD group compared with the sham operation group. In comparison to the VD group, both the SFI and L-NAME groups exhibited a significant decrease in NO content, with a statistically significant difference (P < 0.01). However, there was no significant decrease observed in the NO content of the L-Arg group (Figure 4).

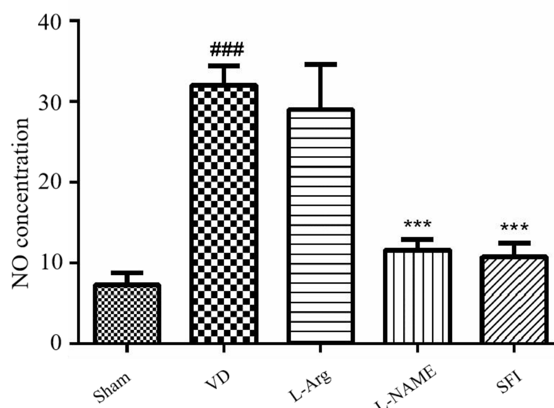

Figure 4 NO content in mice serum in Sham, VD, L-Arg, L-NAME and SFI groups. Compared with the sham group, ###P<0.01. Compared with the VD model group, ***P<0.01, *P<0.05.

3.4 Effect of SFI on hippocampal tissue NOS of VD mice

Compared to the sham operation group, eNOS protein expression in hippocampus was descreaced in VD group (P <0.05),while the protein expression of nNOS and iNOS were significantly increased (P <0.01).In contrast, there were no significant alterations in eNOS, nNOS, and iNOS protein expression in the hippocampus between the VD group and L-Arg group. Notably, both SFI and L-NAME groups exhibited an increase in eNOS protein expression (P <0.05), accompanied by a significant decrease in nNOS and iNOS proteins expression (P <0.05) (Figure 5).

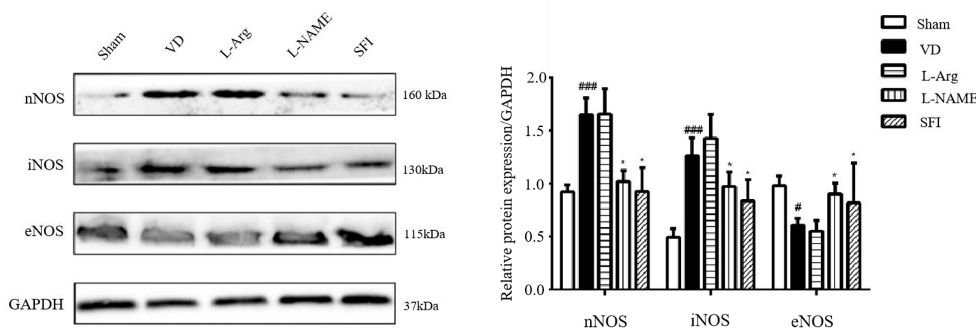

Figure 5 Expression of nNOS, iNOS and eNOS proteins in mouse hippocampus in Sham, VD, L-Arg, L-NAME and SFI groups. Compared with the sham group, #P<0.05, ###P<0.01. Compared with the VD model group, ***P<0.01, *P<0.05.

3.5 Effect of SFI on MDA content, ROS, and GSH activity in the hippocampus of VD mice

As depicted in Figure 6, compared with the sham operation group,the VD group exhibited a significant increase in ROS and MDA levels (P <0.01), along with a notable decrease in GSH activity,and the differences were statistically significant (P <0.01). (P<0.05).Furthermore, both the SFI and L-NAME groups demonstrated a significant reduction in hippocampal ROS and MDA content (P<0.05) as well as an evident enhancement of GSH activity (P<0.05) when compared to the VD group(graph 6).

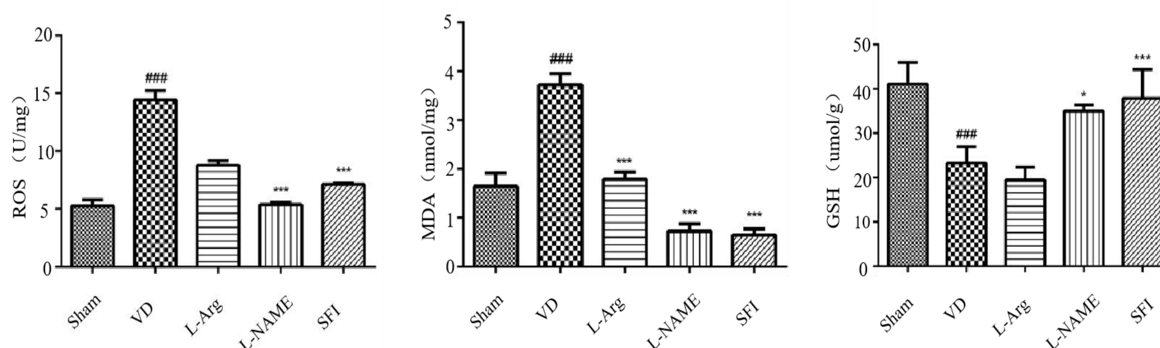

Figure 6 ROS, MDA content and GSH activity in Sham, VD, L-Arg, L-NAME and SFI groups. Compared with the sham group, ###P<0.01. Compared with the VD model group, ***P<0.01, *P<0.05.

4. Discussion

VD is an acquired intelligence injury caused by cerebrovascular disease factors and mainly manifested by cognitive dysfunction.The pathogenesis of VD is complex. Current study mainly concentrated on cholinergic damage, apoptosis, oxidative stress, inflammatory response, excitotoxicity and others[2].

Xu Danyun, et al. [18] have demonstrated that NO is involved in the whole process of VD and acute cerebral ischemia.With the development of cerebral ischemia, glial cell activation triggers the production of inflammatory factors that activate iNOS,resulting in an excessive release of neurotoxic NO. This neuroinflammation subsequently leads to neural cell apoptosis and death [19]. On the other hand, excessive NO can disrupt the regulation of cerebral blood flow, destroy cell membrane structure, compromise BBB function, interfere with DNA transcription and protein synthesis, and directly induce neuronal damage.Ultimately, these effects contribute to cognitive dysfunction in VD [20].

It has been reported that Rg1, the active component of ginseng in SFI, may induce NO [21] production by eNOS through PI3K/Akt pathway, thereby dilating blood vessels during pre-cerebral ischemia and protecting endothelial cells. Meanwhile, Ginsenoside Rh2 not only inhibits NF-κB activation, but also suppresses iNOS activation to reduce NO production and alleviate inflammatory response [22]. Previous studies have demonstrated that SFI can directly scavenge free radicals and peroxides, ensure cerebral blood perfusion, inhibit apoptosis,reduce the degree of CNS lesions in rats with cerebral ischemia and reperfusion injury [6], as well as improve neuronal structural function leading to improved VD outcomes [23-24]. In this study, we observed enhanced performance in water maze positioning, navigation, and spatial exploration tests for both the SFI group and L-NAME group compared to the VD model group. Brain histopathology analysis revealed reduced neuronal damage in the brains of mice treated with SFI or L-NAME under VD conditions; however,no significant improvement was observed in the L-Arginine (L-Arg) group compared to the VD model mice. Furthermore,the NO level was increased in the VD model group while both the SFI group and L-NAME group significantly decreased NO content in VD mice. The NO content of the L-Arginine (L-Arg) group did not differ from that of the VD model group. These findings suggest that SFI can ameliorate pathological tissue damage and cognitive dysfunction in a mouse model of vascular dementia (VD).L-NAME and L Arg serve as inhibitors and precursors of NO synthase, respectively. The beneficial effects of both SFI and L-NAME on cognitive dysfunction may be attributed to their modulation of pathways related to nitric oxide.,

NO is an active gas molecule involved in vasodilation, nerve regulationand immune modulation. Moreover, NO exhibits dual properties as it can both protect blood vessels and exert neurotoxic effects within the nervous system[25]. The three type of nitric oxide synthase (NOS), namely nNOS, iNOS, and eNOS, catalyze the synthesis of NO precursor substances to generate NO. Among these NOS types,endothelial cells predominantly express eNOS which regulates endothelial cell function and cerebral blood flow[26].On the other hand, nNOS is primarily expressed in central and peripheral neurons,where NO derived from nNOS influences synaptic plasticity to promote learning and memory[27]. However, iNOS expression is minimal under normal physiological conditions but can be induced by stimulation from tumor necrosis factor-α (TNF-α) and various cytokines [28]. Prolonged iNOS expression leads to the synthesis of a substantial amount of neurotoxic NO-mediated inflammatory molecules[29-30], resulting in apoptotic damage to hippocampal neurons[31].Excessive NO can interact with O2- to generate peroxynitrite (OONO-) and promote eNOS uncoupling,leading to reduce the activity of MnSOD and glutathione reductase. This results in increased expression of iNOS, creating vicious cycles[32]. Fleszar et al [33] discovered that long-term excessive application of L-Arg leads to excessive NO production, inducing neurotoxicity and damaging nerve cells. However, L-NAME can inhibit neurotransmitter release and the activatio of NOS receptors, regulating NO production against cerebral ischemia excitotoxicity.It protects neurons and improves spatial memory ability in rats. Lin Shaobin, et al.[23-34] indicate that ginsenosides may enhance neurotransmitter content, reduce nerve cell apoptosis,exhibit anti-cerebral ischemia and other effects.They can also decrease the NF-κB phosphorylation response caused by LPS stimulation through intervention in the mTOR pathway, inhibiting iNOS activation and reducing NO release.This ultimately enhances learning and memory ability in rats after ischemia-reperfusion[35].Therefore, inhibiting NOS activation and reducing NO content represent effective strategies for treating VD.Our study found decreased protein expression of eNOS but significantly increased expression of iNOS and nNOS proteins in the VD model group. However, both the SFI and L-NAME group exhibited significant decreases in iNOS and nNOS protein expression while showing an increase in eNOS protein expression compared to the VD mice group.No statistical difference was observed between the L-Arg group's protein expressions compared with those from the VD group. These findings suggest that SFI may modulate NO bioavailability by downregulating iNOS / nNOS while upregulating eNOS to maintain a reasonable level of NO content which alleviates cognitive dysfunction in VD mice.

After cerebral ischemia, BBB injury leads to extravasation of red blood cells and microhemorrhage, resulting in the release of Fe2+ that further generates reactive oxygen species (ROS), thereby contributing to oxidative stress and exacerbating brain tissue damage[36]. Zhang et al.[37] observed a significant reduction in serum superoxide dismutase (SOD) levels and an increase in malondialdehyde (MDA) levels in VD mice However, L-NAME-induced inhibition can improve the oxidative status of the brain and reduce the oxidative stress response in postischemic rats [38]. Gumuslu et al.[39] found that injecting L-NAME into a rat model of global cerebral ischemia,increased SOD activity, decreased lipid peroxide content, and improved the oxidative status after cerebral ischemia. L-Arg serves as a substrate for NOS during NO synthesis by synthase; however, under Arg deficiency conditions, NOS produces O2-. Appropriate physiological concentrations of Arg can reduce O2- generation while excessive L-Arg during cerebral ischemia may worsen dysregulation of NO bioavailability [40].The results from this study demonstrated that SFI and L-NAME enhance GSH activity while reducing ROS and MDA expression levels in VD mice, suggesting that SFI may also exert antioxidative effects by regulating NO bioavailability to ameliorate neuronal impairment and cognitive dysfunction in VD mice.

5. Conclusions

Overall, we demonstrated that SFI can improve cognitive dysfunction in VD mice. the underline mechanism may be related to improved NOS/NO pathway, inhibit oxidative stress, regulate NO bioavailability, affect NO generation, and protect the cerebrovascular nerve units involved.

Acknowledgment

This work was supported by National Natural Science Foundation of China (№.82360880,82060661,81660751), Jiangxi Provincial Natural Science Foundation (№ 20232ACB206057, 20212BAB206092), and Educational Commission of Jiangxi Province of China (№.GJJ218104). Teaching reform research project of Jiangxi Province of China (№JXJG-22-130-1), and Science and Technology Key Project of Fuzhou Medical College (fykj202301).

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