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Original Article

Effects of ginkgo diterpene lactone on brain inflammation and oxidative stress in rats with cognitive impairment of cerebral small vessel disease

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Abstract: Purpose: This study aims to investigate the effects of ginkgo diterpene lactone on brain inflammation and oxidative stress in rats with cognitive impairment of cerebral small vessel disease (CSVD). Methods: We equally assigned 40 Sprague Dawley (SD) rats to the observation group (OG) and the control group (CG) and modeled them for cognitive impairment of CSVD. Rats in OG were given ginkgo diterpene lactone for treatment, while those in CG were injected with the same amount of normal saline. The learning and memorizing ability of rats was tested by the water maze. The oxidative stress and inflammatory response in rats were evaluated. The levels of vascular endothelial growth factor (VEGF) and endostatin (ES) mRNA in the hippocampus of rats were measured. Vascular smooth muscle cells of rats were sampled for cell viability and apoptosis assays. Results: Rats from OG were superior to those from CG in the learning and memorizing ability. After treatment, rats from OG had markedly lower malondialdehyde (MDA) levels and higher levels of superoxide dismutase (SOD), reduced glutathione (GSH), and glutathione peroxidase (GSH-Px) than those from CG (all $P < 0.05$). Concentrations of interleukin-18 (IL-18), transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), and amyloid β-protein 1-40 (Aβ1-40) were markedly lower in OG than in CG (all $P < 0.05$). Rats from OG had markedly higher VEGF levels and lower ES mRNA levels than those from CG (all $P < 0.05$). Cell viability gradually decreased in rats from OG after treatment and was markedly lower than that in rats from CG ($P < 0.05$). Cell apoptosis rate was markedly higher in OG than in CG ($P < 0.05$). Conclusion: Ginkgo diterpene lactone can inhibit oxidative stress and inflammatory response in rats with cognitive impairment of CSVD to a certain degree.

Keywords: Ginkgo diterpene lactone, cognitive impairment of cerebral small vessel disease, inflammation, oxidative stress

Introduction

As a common cerebrovascular condition among the middle-aged and elderly people, cerebral small vessel disease (CSVD) has an extraordinarily high global incidence [1]. What's worse, amid the accelerated population aging, its incidence is on a rising trend [2]. According to statistics, the current incidence of CSVD is as high as 3-8% [3], but its pathogenesis is still unclear [4]. In clinical practice, a complete cure for CSVD is quite difficult, and its symptoms can only be controlled through the long-term maintenance treatment [5]. Cognitive impairment at varying degrees is easily induced in patients during CSVD progression [6], which seriously affects the life quality and health of patients. Therefore, it marks one of the focuses of current clinical research to develop effective treatment strategies for CSVD.

Ginkgo diterpene lactone, a common Chinese patent medicine, can suppress the platelet-activating factor [7]. It mainly inhibits platelet aggregation and oxygen free radicals, commonly used for cerebral ischemia reperfusion injury [8]. Previous studies suggest that ginkgo diterpene lactone is effective in the treatment of acute cerebral infarction, cerebral atherosclerosis, and ischemic stroke [9-11]. But its application in CSVD is rarely studied. To our knowledge, ginkgo diterpene lactone can achi-

eve marked treatment responses in patients with cerebrovascular diseases, so we speculate that it may also be effective in CSVD treatment. To verify our hypothesis, we injected ginkgo diterpene lactone into animal models of cognitive impairment of CSVD and evaluated the oxidative stress response in rat brain tissues, seeking to provide a reliable theoretical basis for treating CSVD with ginkgo diterpene lactone.

Materials and methods

Information of animals

Forty Sprague Dawley rats (in a 1:1 sex ratio) were purchased from Beijing Wanhe Technology Co., Ltd. (license number: SYXK (Beijing) 2020-0008), weighing 320-360 g. Rats were raised at 22-25°C with 50-65% humidity, 5 rats per cage, with no limits on food and water intake. This study has been approved by the animal ethics committee of our hospital.

Methods of modeling

All rats received carotid artery ligation for CSVD modeling. After one week of adaptive feeding, they were anesthetized by an intraperitoneal injection of 10% chloral hydrate (4 mL/kg) and were put in a supine position. A 2-cm cut was made at the center of the neck to separate the left common carotid artery. Caution was required during the operation to avoid damage to the muscles, blood vessels, and nerve tissues of rats. After the operation, the wound was sutured, and all rats were kept in cages. One week later, ligation of the right carotid artery was performed on all rates following the same procedures as above. Hole-digging ability was tested 4 weeks after the successful ligation of the arteries on both sides. Successful modeling was determined if the modeled rats showed a remarkably different hole-digging ability compared with normal rats.

Animal grouping

Totally 40 modeled rats were randomly and equally divided into the observation group (OG) and the control group (CG). After the hole-digging test, rats from OG were given an intraperitoneal injection of ginkgo diterpene lactone solution (1.5 mg/mL) at a dose of 2 mL/kg for consecutive 14 days. The solution was comprised of ginkgo diterpene lactone (3 mg/kg) and 0.9 sodium chloride solution. Rats from CG were injected with the same amount of normal saline.

Water maze test

All rats were placed in the Morris water maze, a round pool with a diameter of 120 cm, to test their learning and memorizing ability. With an appropriate amount of water at 25°C, the pool was divided into 4 quadrants marking east, south, west, and north separately. Each quadrant contained a transparent platform away from the wall of the pool. The moving track of the rat was recorded by a machine. 1. Navigation time: Rats were placed in each quadrant respectively and their movement trajectory and escape latency within 2 min were recorded. The test was repeated multiple times to obtain the average value. 2. Space exploration: The day after navigation task, rats were placed in the pool again to record their frequency of reaching the platform within the specified time. The test was repeated multiple times to obtain the average value.

PCR detection of VEGF and ES mRNA levels

Rats were sacrificed and then the total mRNA was isolated from the lysed hippocampus tissue of dead rats using the RNA extraction kit. The purity, concentration, and integrity of total mRNA were measured by UV spectrophotometer and agarose gel electrophoresis. Then RNA was reverse transcribed into cDNA, followed by the amplification according to the kit instructions. Vascular endothelial growth factor (VEGF): forward primer: 5'-CATAGCTTGAACGACG-3'; reverse primer: 5'-GCTAGGCTTCGGAGAGACC-3'. Endostatin (ES): forward primer: 5'-TCCACCCGCTACGACCACCT-3'; reverse primer: 5'-CCATAGCCACCATCGCACCGTGG-3'. PCR conditions: pre-denaturation at 94°C for 30 sec, followed by 40 cycles of denaturation at 94°C for 5 sec, annealing at 60°C for 15 sec, and extension at 60°C for 10 sec. Three replicate wells were set for each sample and the experiment was performed in triplicate. The experimental data was analyzed with $2^{-\Delta\Delta qct}$.

Oxidative stress response

Before and after treatment, the reduced glutathione (GSH), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) levels in rats

were determined on an automatic biochemical analyzer, and the malondialdehyde (MDA) levels were measured by the thiobarbituric acid test.

Inflammatory response

The concentrations of interleukin-18 (IL-18), transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), and amyloid β-protein 1-40 (Aβ1-40) were measured by the enzyme-linked immunosorbent assay. The IL-18 kit was purchased from Shanghai Xinyu Biotechnology Co., Ltd., Cat. No. IL-18BP; the TGF-β1 kit was bought from Jiangxi Iboin Biotechnology Co., Ltd., Cat. No. IB-E10088; the TNF-α kit was provided by Shanghai Walan Biotechnology Co., Ltd., Cat. No. ABE10038; the Aβ1-40 kit was purchased from Wuhan Yipu Biotechnology Co., Ltd., Cat. No. CK-E11681.

Cell information and culture

Vascular smooth muscle cells were collected from sacrificed rats. Next, they were cultured with a Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum at 37°C in a humid atmosphere with 5% CO_2.

MTT assay for cell proliferation

Cells were seeded in a 96-well plate (4×10^3 cells/well) and incubated at 37°C. At 24 h, 48 h, 72 h, and 96 h, we added 20 μL MTT solution (5 μmg/mL) to the plate and inoculated at 37°C for 4 h. Then, 200 μL dimethyl sulfoxide was added to each well. Finally, the OD values were measured at 490 nm using a V-1200 spectrophotometer.

Detection of cell apoptosis

Cells were digested with 0.25% trypsin and then washed twice with PBS. Next, they were mixed with 100 μL binding buffer to prepare a suspension at a density of 1×10^6 cells/mL and then incubated together with Annexin V-FITC and PI at room temperature in the dark for 5 min. Finally, cell apoptosis was detected using the FC500MCL flow cytometry system.

Statistical analysis

Data analysis was performed using SPSS20.0 and data visualization using GraphPad Prism 7. The measurement data were expressed by the mean ± standard deviation (mean ± SD). The inter-group comparison was analyzed by the independent sample t-test, denoted by t. The comparison between multiple groups was analyzed by the one-way ANOVA and the post hoc pairwise comparison was performed by the LSD-t-test. Multiple time points were compared by the repeated measures ANOVA. A statistical difference was determined when $P < 0.05$.

Results

Comparison of learning and memorizing ability of rats

According to the results of the Morris water maze test (**Figure 1**), the escape latency during navigation task of rats was markedly shorter in OG than in CG ($P < 0.05$), the path length was markedly shorter in OG than in CG ($P < 0.05$), and the number of times for them to reach the platform during space exploration was markedly higher in OG than in CG ($P < 0.05$).

Comparison of oxidative stress response in rats

The levels of oxidative stress response indexes in rats after treatment were tested. As shown in **Figure 2**, rats in OG had markedly lower MDA levels but markedly higher levels of SOD, GSH, and GSH-Px than rats in CG ($P < 0.05$).

Comparison of inflammatory response in rats

The concentrations of inflammation markers (IL-18, TGF-β1, TNF-α, and Aβ1-40) after treatment were markedly lower in rats from OG than those from CG ($P < 0.05$) (**Figure 3**).

Comparison of expression of VEGF and ES mRNA in the hippocampus of rats

According to PCR test results (**Figure 4**), rats from OG had markedly higher VEGF levels but markedly lower ES mRNA levels than those from CG (all $P < 0.05$).

Comparison of vascular smooth muscle cell proliferation and apoptosis in rats

Vascular smooth muscle cells were collected from sacrificed rats and then cultured for the test of cell proliferation and apoptosis by the MTT assay. As shown in **Figure 5**, cell viability

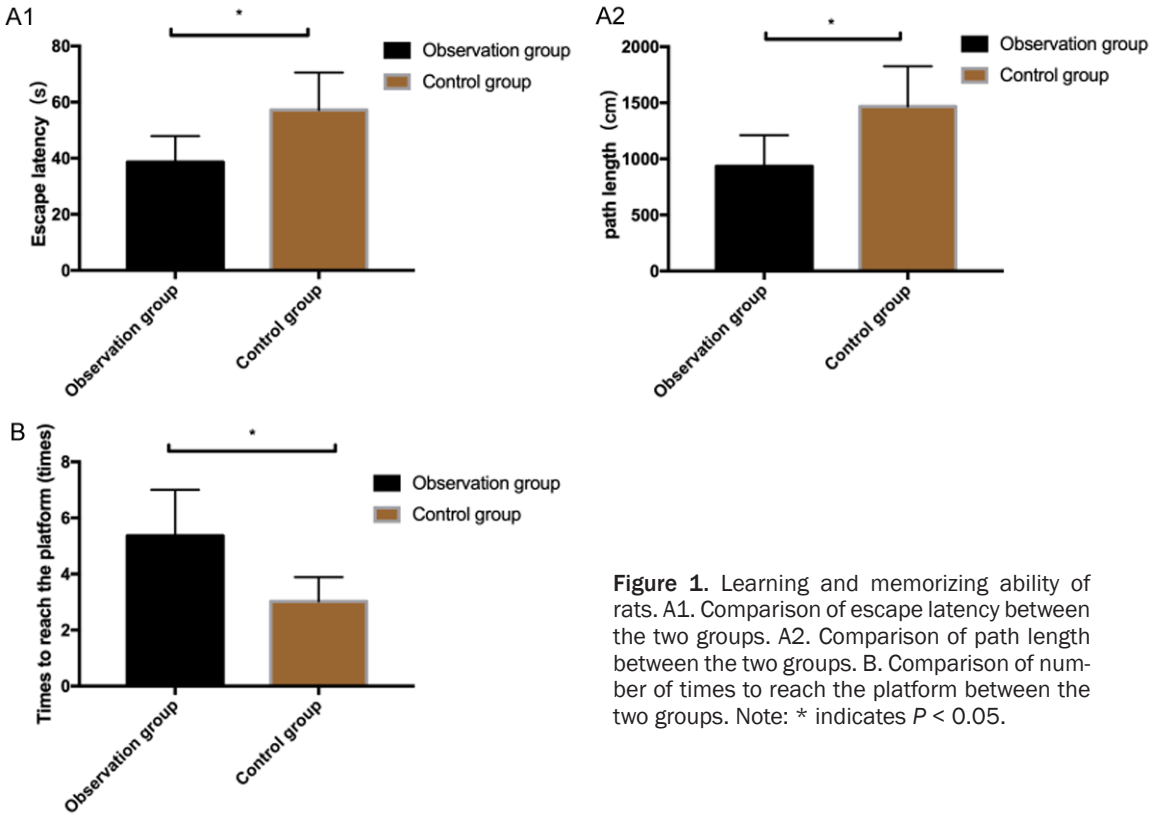

Figure 1. Learning and memorizing ability of rats. A1. Comparison of escape latency between the two groups. A2. Comparison of path length between the two groups. B. Comparison of number of times to reach the platform between the two groups. Note: * indicates $P < 0.05$.

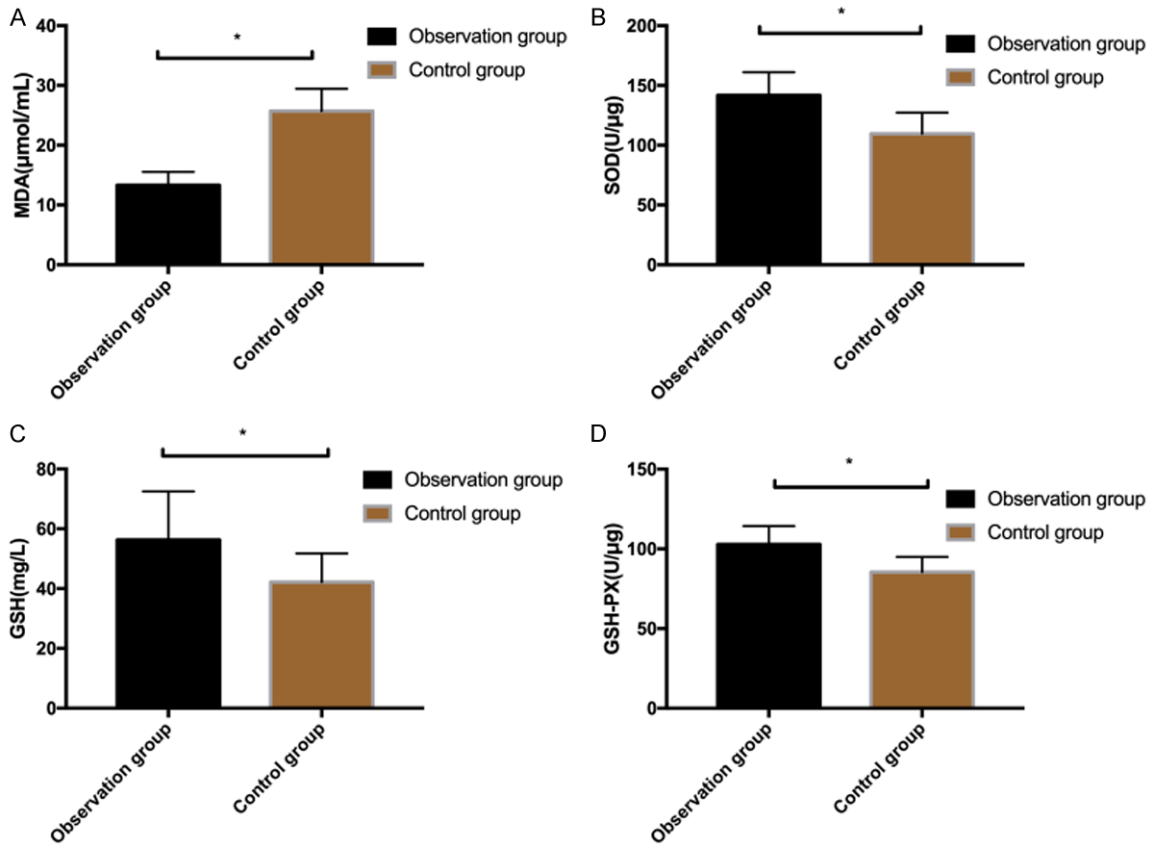

Figure 2. Expression of oxidative stress response indexes in rats. A. Comparison of MDA levels between the two groups. B. Comparison of SOD levels between the two groups. C. Comparison of GSH levels between the two groups. D. Comparison of GSH-Px levels between the two groups. Note: * indicates $P < 0.05$.

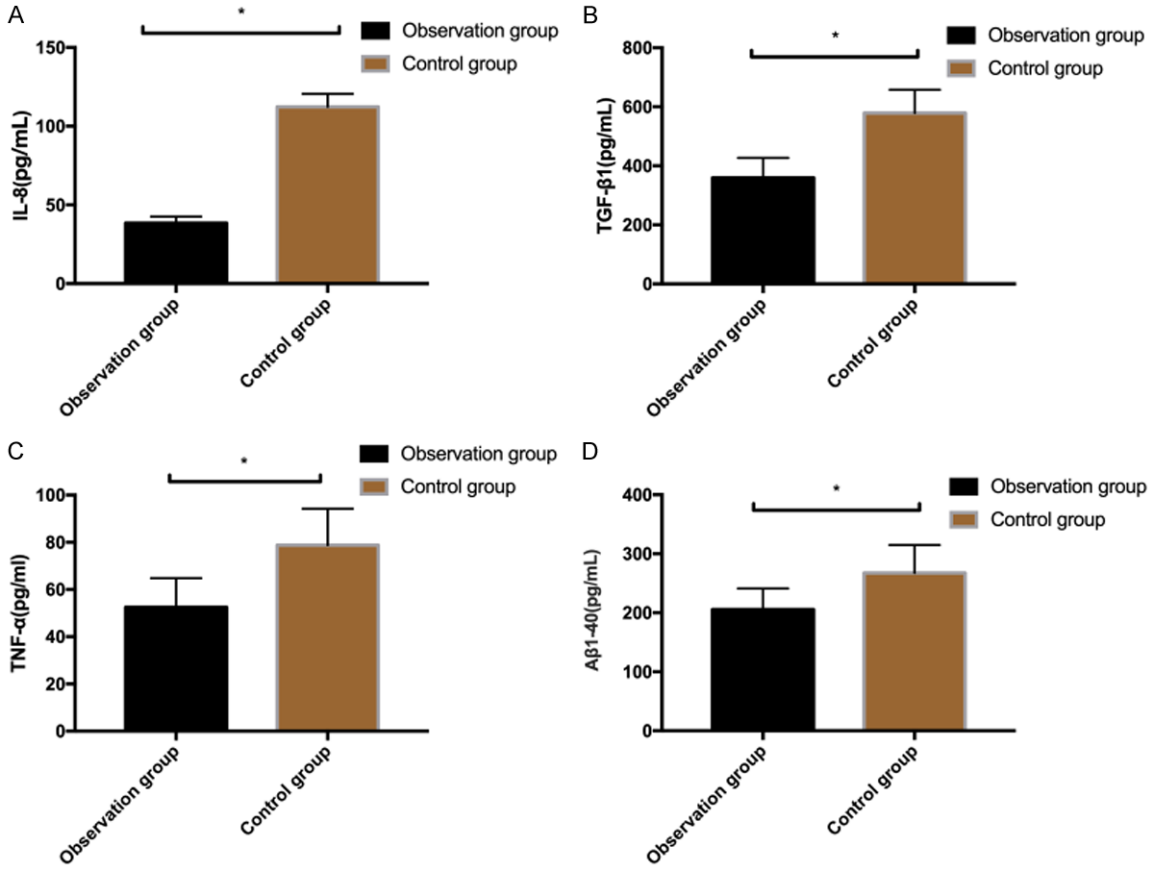

Figure 3. Concentrations of inflammatory response indicators in rats. A. Comparison of IL-18 levels between the two groups. B. Comparison of TGF-β1 levels between the two groups. C. Comparison of TNF-α levels between the two groups. D. Comparison of Aβ1-40 levels between the two groups. Note: * indicates $P < 0.05$.

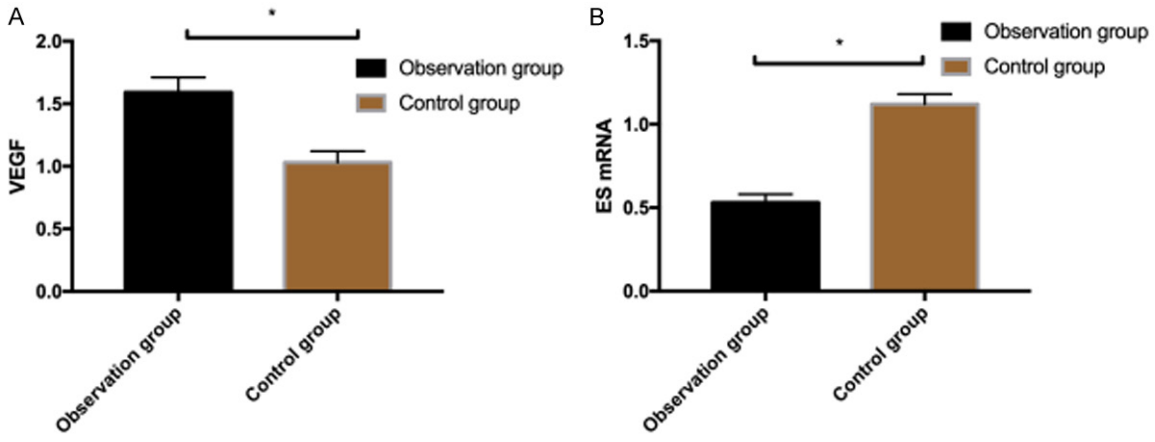

Figure 4. Expression of VEGF and ES mRNA in the hippocampus of rats. A. Comparison of VEGF levels in the hippocampus of rats between the two groups. B. Comparison of ES mRNA levels in the hippocampus of rats between the two groups. Note: * indicates $P < 0.05$.

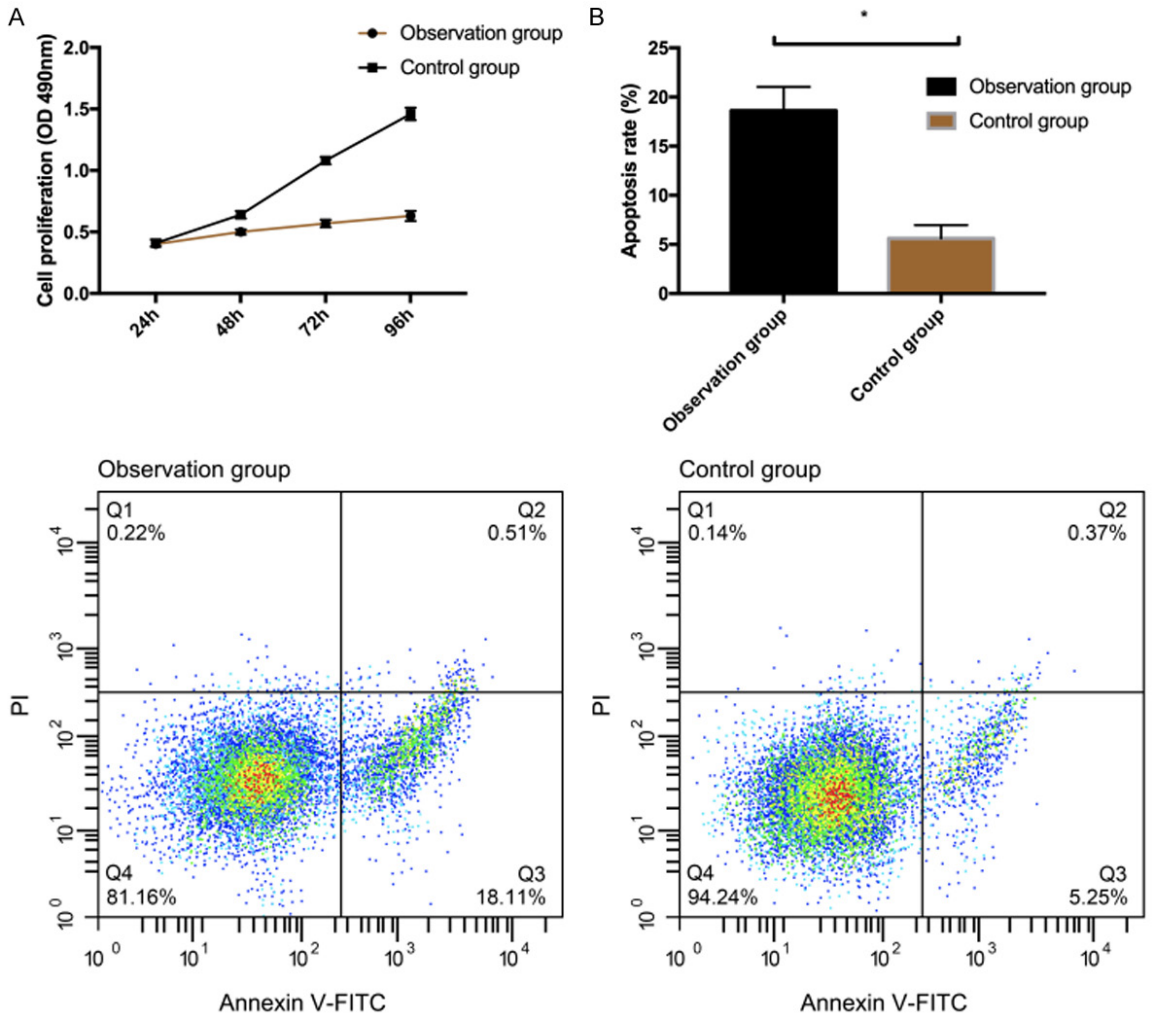

Figure 5. Proliferation and apoptosis of vascular smooth muscle cells in rats. A. Comparison of vascular smooth muscle cell proliferation in rats between the two groups. B. Comparison of vascular smooth muscle cell apoptosis rate in rats between the two groups. Note: * indicates $P < 0.05$.

gradually decreased in rats from OG after treatment and was markedly lower than that in rats from CG ($P < 0.05$), and cell apoptosis rate was markedly higher in OG than in CG ($P < 0.05$).

Discussion

Cerebrovascular diseases have always been the main cause of death and dysfunction worldwide [12]. Among them, CSVD, generally diagnosed in the elderly by the neuroimaging results, is regarded as the main vascular factor leading to dementia, cognitive decline, gait disturbance, emotional disorder, and stroke [13, 14]. CSVD plays a vital role in lacunar infarction and deep or cortical hemorrhage [15]. Therefore, effective control of CSCD progression has been the focus of clinical research. Ginkgo diterpene lactone is effective in a variety of cerebrovascular diseases [16], but its treatment efficacy in CSVD has not been fully understood. This study investigated the effects of ginkgo diterpene lactone on brain inflammation and oxidative stress in rats with cognitive impairment of CSVD.

The learning and memorizing ability of rats were tested by the Morris water maze. Rats treated with ginkgo diterpene lactone had markedly shorter escape latency during navigation task, significantly shorter path length, and remarkably more times of reaching the platform during space exploration than rats injected with normal saline. Such results indicate that our animal modeling was successful and imply that ginkgo diterpene lactone can effec-

tively improve the learning and memorizing ability of rats with cognitive impairment of CSVD. The Morris water maze is a commonly used method to test learning and memorizing ability, which can intuitively reflect the formation of learning and memory in rats [17]. A previous study suggests that ginkgolide has an excellent treatment efficacy for cerebrovascular diseases [18], which is similar to the results of our study. We speculate that ginkgolide can unclog small vessels and improve cognitive function due to its role in anti-inflammation, protection of central nervous system, anti-platelet aggregation, and anti-atherosclerosis. Expression of oxidative stress response indicators in rats after treatment was tested. Rats in OG had markedly lower MDA levels and markedly higher levels of SOD, GSH, and GSH-Px than those in CG, indicating that ginkgo diterpene lactone can reduce the oxidative stress response and improve the activity of daily living of rats with CSVD. Oxidative stress refers to a state of imbalance between oxidation and antioxidant effects in the body [19]. It is a negative effect produced by free radicals and is an important factor for aging and diseases [20]. It can be seen that the antioxidant function of ginkgo diterpene lactone has a positive therapeutic effect on CSVD rats. The concentrations of inflammation markers (IL-18, TGF-β1, TNF-α, and Aβ1-40) after treatment were markedly lower in rats from OG than those from CG, indicating that ginkgo diterpene lactone can inhibit inflammation caused by CSVD. Inflammatory factors are involved in various diseases in the human body [21]. Reducing the inflammatory response is favorable for anti-infection and immune regulation and can indirectly lead to changes in cytokine levels [22]. Rats from OG had markedly higher VEGF levels and markedly lower ES mRNA levels in the hippocampus than rats from CG. VEGF is highly specific to increase vascular permeability, and promote extracellular matrix degeneration, vascular endothelial cell migration, proliferation, and angiogenesis [23, 24]. ES is the strongest experimental tumor angiogenesis inhibitor [25]. It can inhibit the growth of blood vessels [26]. The results of this study illustrate that ginkgo diterpene lactone can repair damaged blood vessels and promote vascular regeneration. To further explore the effect of ginkgo diterpene lactone on CSVD, we sacrificed the rats and collected their vascular smooth muscle cells to test cell viability and apoptosis. Cell viability gradually decreased in rats from OG after treatment and was markedly lower than that in rats from CG, and the cell apoptosis rate was markedly higher in OG than in CG. Such results reflect the application value of ginkgo diterpene lactone in CSVD. In previous studies, ginkgo diterpene lactones have been found to protect neurons by inhibiting platelet aggregation and preventing thrombosis through the phosphoinositol 3 kinase-serine/threonine protein kinase (PI3K-Akt) pathway. The occurrence of cerebrovascular disease is relevant to the decrease of hemodynamics and vascular obstruction [28]. Therefore, we speculate that ginkgo diterpene lactone can reduce the process of inflammatory injury and oxidative stress in cerebrovascular tissue by reducing platelet aggregation and improving cerebrovascular hemodynamics, so as to achieve the purpose of treating cerebrovascular disease.

Of course, there are still many limitations in this study. For example, the specific therapeutic mechanism of ginkgo diterpene lactone on CSVD remains to be explored. In addition, we were unable to evaluate the long-term prognosis of patients due to the short duration of the experiment. Moreover, the small number of cases may make some statistical calculations of the results haphazard. Therefore, we need to conduct more complete experimental analysis in the future to address all the above limitations.

In summary, ginkgo diterpene lactone can inhibit oxidative stress and inflammatory response in rats with cognitive impairment of CSVD to a certain degree.

Disclosure of conflict of interest

None.

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