

The improvement and mechanism of *Scutellaria baicalensis* Georgi stems and leaves flavonoids on okadaic acid-induced learning and memory impairment in rats

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Abstract

Background

AD is a progressive neurodegenerative disease that seriously threatens the physical and mental health for the elderly. The disease is pathologically associated with A β plaque deposition, tau hyperphosphorylation, cholinergic damage, oxidative stress and neuroinflammation. okadaic acid (OA), an inhibitor of protein phosphatases PP1 and PP2A, can reduce the dephosphorylation of phosphorylated tau protein and result in the hyperphosphorylation of tau protein, which in turn leads to NFT deposition and indirectly induces AD. Therefore, the brain injury in mimic AD model established by intracerebroventricular injection of OA conforms to the pathogenesis of AD in rats.

Objective

To investigate the effect and mechanism of *Scutellaria baicalensis* Georgi stems and leaves flavonoids (SSFs) on learning and memory impairment induced by intracerebroventricular injection of OA in rats.

Methods

The mimic AD model of memory impairment of rats was established by intracerebroventricular injection of OA. Morris water maze was to screen the successful model of memory impairment. The successful model rats were randomly divided into model group, three doses of groups for SSFs 25, 50, and 100 mg/kg, and positive drug Ginkgo biloba tablet (GLT) 200 mg/kg group. The rats' learning and memory abilities were detected with the Morris water maze. The morphological changes of nerve cells were observed by hematoxylin-eosin (HE) staining. The Choline acetyltransferase (ChAT) protein expressions were detected with immunohistochemistry (IHC). The phosphorylation level of tau protein at Ser262 and Ser396 sites was assayed with Western blotting (WB). The activities of inducible nitric oxide synthase (iNOS) and constitutive nitric oxide synthase (cNOS) were measured with ultraviolet spectrophotometry. Enzyme-linked immunosorbent assay (ELISA) was used to detect the level of inflammatory factors interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6).

Results

The intracerebroventricular injection of OA caused the rats' learning and memory impairment and neuropathological changes. The protein expression of ChAT was reduced ($P < 0.01$) and the phosphorylation levels of tau protein at Ser262 and Ser396 were significantly increased ($P < 0.01$), the activity of iNOS increased ($P < 0.01$) and cNOS decreased ($P < 0.05$) in the hippocampus and cerebral cortex. The levels of IL-1 β and TNF- α in the cerebral cortex were increased ($P < 0.01$), and the level of IL-6

was decreased ($P < 0.05$). The three doses of SSFs and GLT treatment to rats can differently ameliorate the above abnormal changes caused by OA.

Conclusion

SSFs can improve the rats' learning and memory impairment caused by intracerebroventricular injection of OA and the effective mechanism may be related to the positive regulation in ChAT protein expression, tau hyperphosphorylation, NOS activity, and inflammatory cytokine levels.

Background

Alzheimer's disease (AD) is an age-related progressive central nervous system disease characterized by memory cognitive impairment, behavioral abnormalities, and personality changes [1]. Pathologically, AD is characterized by senile plaques (SP) formed by extracellular beta-amyloid protein ($A\beta$) deposition and neurofibrillary tangles (NFTs) caused by intracellular tau hyperphosphorylation [2]. Protein phosphatase PP1 and PP2A can dephosphorylate of phosphorylated tau protein to lower tau protein hyperphosphorylation. okadaic acid (OA) is a polyether monocarboxylic acid compound that can bind to the catalytic subunits of protein phosphatases of PP1 and PP2A and inhibit their activity and then, suppress the tau dephosphorylation. Therefore, the hyperphosphorylated tau protein aggregates to form NFTs and produce AD-like neurological damage and dysfunction [3, 4]. Then, any strategies that can inhibit the toxicities of OA are beneficial to the treatment of AD.

SSFs are flavonoids isolated from the aerial parts of *Scutellaria baicalensis* Georgi, with a variety of pharmacological activities [5]. Previous studies in our laboratory have confirmed that SSFs can improve memory impairment, inhibit neuronal apoptosis, promote nerve regeneration, and effectively inhibit tau phosphorylation in mimic AD model induced by $A\beta$, $AlCl_3$ and D-galactose [6–9]. It also has a good improvement on OA-induced the less Nissle in neuron, astrocytes, and oxidative stress in the brain [10]. However, the effects of SSFs on OA-induced the rats' memory impairment and its regulatory mechanism have not been reported. In the present study, we established the mimic AD rats' model by intracerebroventricular injection of OA to investigate the effect and mechanism of SSFs on learning and memory impairment and related to the effective mechanism.

Materials

Animals

Sixty healthy male Sprague Dawley rats (300-350g, 8–9 weeks) were purchased from the Experimental Animal Center of Hebei Medical University Co., Ltd. (Certification No. SCXK (Ji) 2018 – 1003). Before the operation, all the rats were housed in the SPF-grade Experimental Animal Center of Chengde Medical College at a constant temperature ($24 \pm 1^\circ C$) and humidity ($50 \pm 10\%$). The experimental protocol abided

by the relevant provisions of the "Guidelines for Animal Ethics and Welfare" and was approved by the Animal Ethics Committee of Chengde Medical College [No. CDMU-LAC-201800313013].

Drug and reagents

As shown Fig. 1, *Scutellaria baicalensis* Georgi stems and leaves flavonoids (SSFs) were provided by Institute of Traditional Chinese Medicine, Chengde Medical College, with a purity of 87.11%, and stored at 4°C. OA was purchased from Sigma Co., Ltd. GLT (Specification: 200 mg/tablet) was supplied by Guizhou Xinbang Pharmaceutical Co., Ltd., China. Anti-Tau-pSer262 antibody (Lot. BS194P) and Anti-Tau-pSer396 antibody (Lot. BS4196) were purchased from Bioworld Technology Inc. USA. Anti-nNOS antibody (Lot. BS-0156R) and Anti-iNOS antibody (Lot. BS-0162R) were purchased from Beijing Bioss Biological Technology Co., Ltd., China. Anti-ChAT antibody (Lot. AO1192-5) and Ellisa kits of IL-1 β (Lot. RK00009), TNF- α (Lot. RK00029) and IL-6 (Lot. RK00020) were bought in Boster Biological Technology Co., Ltd., China.

Methods

Experiment designed

As shown in Fig. 2, the experiment was consecutively performed 60 days. 52 rats were intracerebroventricularly injected OA and 8 rats given the same surgery with intracerebroventricular injection of 10% DMSO by same volume were as sham-operated group. All rats have been recovery for 20 days of operation. On day 22 after operation, the successful mimic AD model in memory impairment was screened with Morris water maze test. The successful mimic AD model rats were administered three doses of SSFs or GLT from day 25 to 60 of operation and the rats' learning and memory abilities were conducted during 53 to 59 of operation period administration. On day 60 of operation, the rats were anesthetized with isoflurane and decapitated for preparing the samples

Preparation of mimic AD model in rats

The rats were performed the intracerebroventricular injection of OA to establish memory impairment model for mimic AD. The rats were anesthetized with 10% chloral hydrate (300 mg/kg) for operation after the adaptive feeding for 1 week. Then, the rats' head was fixed on the brain stereotaxic instrument and cut through a median incision and the subcutaneous tissue was gently separated. The anterior fontanel of the skull was fully exposed to the posterior 1 cm of the herringbone suture. According to the rat brain stereotaxic map, a hole was drilled at 0.8mm behind the anterior fontanel and 1.3mm on the right side of the sagittal suture. The lateral ventricle injection (depth 3.5mm) was performed at the drilling site. The rats in OA group were randomly selected and slowly injected 1.5 μ L OA (200 ng/kg) with duration 5 min and retained needle for 10 min. After 3 days, the same procedure was performed once again. The rats in sham-operated group were intracerebroventricularly injected the equal volume of 10% DMSO like above procedure.

Morris Water Maze Device and Determination Method

The Morris water maze of rats was a stainless-steel circular pool with a diameter of 120 cm and a height of 50 cm and a height of 30 cm platform. The pool was artificially divided into 4 quadrants with 4 water entry points (2 distal points and 2 proximal points). Before the beginning of the test, a small amount of black edible melanin was added to the water maze to let the water with black and the water temperature was kept at $(23 \pm 1) ^\circ\text{C}$. The water surface was 1.5 cm higher than the platform (hidden platform), and all signs around the maze remained unchanged throughout the testing. Image acquisition and analysis system was used to record the rats' swimming trajectory and analysis data of rats' behavior in the Morris water maze. On day before training, each rat was allowed to swim freely in the water maze for 120 s to familiarize the water environment, and the training began on the second day and the rats were trained twice a day. On the first trial, the rats were placed into the water at the distal point by facing the pool wall to look for the hidden platform. The acquisition system recorded the time spent of rats to find the hidden platform (latency). If the rats found the hidden platform within 60 s, the rats were had a rest for 20 s on the hidden platform. If the rats did not find the hidden platform within 60 s, the rats were guided onto the hidden platform and stayed there for 20 s, and the latency was recorded 60 s. On the second trial, the rats were put into the water from the proximal point to search for the hidden platform. The average latency of the 2 trails was as the academic performance of the rats on the day.

Successful mimic AD model screening, grouping and administration

During the day 22 ~ 25 of the operation, the Morris water maze was used to screen mimic AD model in memory impairment for the intracerebroventricular injection of OA rats. The hidden platform was placed in the second quadrant (Q2), which was posited as the target quadrant. The training was consecutively carried out 4 days with 2 trials a day for screening the rats' mimic AD model of learning and memory impairment. The data of rats' training on the day 4 was calculated the successful mimic AD model. On the day 4 of water maze training, the latency of finding the hidden platform of each rat with the intracerebroventricular injection of OA was A, the average latency of finding the hidden platform of rats in the sham-operated group was B. The screening ratio (SR) = $(A-B) / B$. When the SR of any rat with the intracerebroventricular injection of OA was greater than 0.2, this rat was confirmed as the successful mimic AD model rat. The successful model rats were randomly divided into 5 groups, model group, three doses of drug groups, and positive control group. The rats in the drug group were intragastrically administered 25, 50 and 100 mg/kg SSFs and the rats in the positive control group were intragastrically administered 200 mg/kg GLT for 36 days. The sham-operated group and the model group were orally given an equal volume of distilled water.

Detection of the rats' learning and memory impaired using the morris water maze test

On the day of 29 of administration, correspond to the day 53 of operation, the learning and memory ability of the rats were tested with Morris water maze after intragastric administered 60 min. The detection is divided into two days test of positioning navigation trial for the rats' memory acquisition ability, one day test of probe trial for the rats' memory retention ability, three days test of the reversal trial for rats' memory re-learning ability, and one day of visible platform trial for rats' swimming speed.

Determination of the rats' memory acquisition ability

The positioning navigation trial was used to determine the memory acquisition ability of rats. The memory acquisition test was performed on the day 29–30 of administration, corresponding to the day 53–54 of operation. The hidden platform was placed the same position (Q2) as the model screened procedure. The latency of the rats to find the hidden platform from the water was recorded. The average latency of the two trials was used to calculate the learning and memory performance of rats on the day.

Determination of the rats' memory retention ability

The probe trial was used to determine the rats' memory retention ability on day 31 after administration, corresponding to the day 55 after operation. The hidden platform was removed out of the pool and the rats were allowed swimming in the water for 60 s to search for the hidden platform. The swimming time and trajectory of the rats spent in the target quadrant (Q2) within 60 s, where the hidden platform was located, was recorded as the test score of the rats on the day.

Determination of the rats' memory retrieval ability

The reversal test was used to detect the rats' memory retrieval ability on the day 32–34 of administration, corresponding to the day 56–58 after operation. The hidden platform was moved to the opposite side of the target quadrant (Q4). The latency of the rats to find the hidden platform was recorded and the average latency of the two trials was taken as the test result of the rats on the day.

Determination of the rats' swimming speed

The visible platform test was used to determine the swimming speed of rats. In order to exclude the influence of physical and visual differences on swimming speed, the visible platform test was carried out on the day 35 of administration, corresponding to the day 59 of operation. The platform was higher 1.5 cm of the water surface and the latency of rats onto the visual platform was recorded. The average latency of the two trials was used as the test performance of the rats on the day.

Sample preparation

On the day 36 of administration 60 min, corresponding to the day 60 of operation, the rats were anesthetized with isoflurane and decapitated. The 3 rats' brain of every group was fixed with 4% formaldehyde for 24 h and embedded in paraffin for HE staining and immunohistochemistry assay. The remained brains were rapidly placed on the ice and gently isolated hippocampus and cerebral cortex. These separated brain tissue were frozen in a refrigerator at 80°C for the measurement of Western blotting, UV spectrophotometry and ELISA kits.

Morphological changes of neurons in the hippocampus and cerebral cortex detected by HE stained

The paraffin of brain tissue was cut slices with 5 μm and perform the routine procedure by HE stained. The morphological changes of neuron in the hippocampus and cerebral cortex were observed taken picture under an optical microscope by a person unfamiliar to the experimental design. Meanwhile, the perfect neuron number was also counted under 9 visible regions of 3 independent samples ($n = 3$) by 400 magnifications.

Determination of ChAT protein expression in the hippocampus and cerebral cortex by immunohistochemistry method

Paraffin sections were routinely performed by immunohistochemical staining. The concentration of ChAT antibody was in 1:200, and phosphate buffered solution (PBS) was used as a negative control. The cytoplasm or projections was stained with brownish-yellow as positive expression of ChAT protein. The photographs were analyzed with Image J Medical Image Analysis System and taken picture by 400 magnifications.

Measurement of Tau-pSer262 and Tau-pSer396 in the hippocampus detected by Western blotting method

The frozen hippocampus tissue was lysed with RIPA buffer. The protein concentration was determined by the BCA method. The protein was separated by SDS-PAGE gel electrophoresis and the PVDF membrane was transferred. This PVDF membrane was sealed with the milk powder, and then added the primary and secondary antibodies of the target protein. The ultra-sensitive ECL chemiluminescence solution was exposed and developed, and the protein bands were analyzed by Image J Software.

Determination of iNOS and cNOS in the hippocampus and cerebral cortex by UV spectrophotometry method

The frozen hippocampus or cerebral cortex were grinded and 10% homogenate supernatant was used to assay the activities of iNOS and cNOS at $\lambda = 530\text{nm}$. The measured procedure was based on the kit instructions.

Detection of inflammatory factor IL-1 β , TNF- α and IL-6 levels in cerebral cortex by ELISA method

10% homogenate supernatant of cerebral cortex was used to detect the levels of IL-1 β , TNF- α and IL-6. The detected procedure was based on the ELISA kit instructions.

Statistical Analysis

Data were analyzed using SPSS 21.0 Statistical Software, and expressed as Mean $\pm$ Standard Deviation (mean $\pm$ SD). Two or One-way ANOVA was used to compare the means among multiple samples. $P < 0.05$ was considered statistically significant.

Results

Screening ratio of successful mimic AD model of rats in learning and memory impairment

Figure 3 is the SR of successful mimic AD model of rats in learning and memory impairment. With prolonging the training days, the time of finding the hidden platform in each group was gradually shortened. There was no significant difference in the latency between the groups in the first 3 days of the test ($P > 0.05$). On the day 4 of the training, the latency of OA group was significantly longer than that of the sham-operated group by 1.41 times ($P < 0.05$). According to the results on the day 4 of Morris water maze training, the SR of this experiment was 76.92%. (Fig. 3).

The effect of SSFs on rats' learning and memory impairment induced by OA

The effect of SSFs on OA-induced the rats' learning and memory acquisition ability impairment

Figure 4 is the result of the rats' memory learning and memory acquisition ability on day 1–2 of the Morris water maze test, which was the day 29–30 of administration, correspond to the day 53–54 of operation. The data showed that intracerebroventricular injection of OA can reduce the rats' learning and memory acquisition ability. Compared with the sham-operated group, the latency of rats in the model group to find the hidden platform increased by 0.04 and 3.32 folds respectively, on d1 and d2 of Morris water maze test (all $P < 0.01$). However, three doses of SSFs can improve OA-induced the rats' learning and memory acquisition deficits. For the first day of the learning and memory acquisition ability test, three doses of SSFs and GLT 200 mg/kg all can shorten the latencies to find the hidden platform induced by OA, but there were not significant than those of model group ($P > 0.05$). For the second day of the learning and memory acquisition ability test, three doses of SSFs decreased the latency of model group rats to find the hidden platform by 49.68% (25 mg/kg SSFs, $P > 0.05$), 63.51% (50 mg/kg SSFs, $P < 0.05$) and 46.56% (100 mg/kg SSFs, $P < 0.01$), respectively. GLT 200 mg/kg also appeared the shorter latency of model group rats to find the hidden platform by 39.03% ($P < 0.05$).

The effect of SSFs on OA-induced memory retrieval ability disorder in rats

Figure 4 is the result of the rats' memory retrieval ability on day 4–6 of Morris water maze test, which was the day 32–34 of administration, correspond to the day 56–58 of operation. The data showed that intracerebroventricular injection of OA can decrease the memory retrieval ability of rats. Compared with the sham-operated group, the latency of rats in the model group prolonged by 147.42% (4 d, $P < 0.01$), 84.81% (5 d, $P < 0.01$) and 85.70% (6 d, $P < 0.01$), respectively. However, the three doses of SSFs can ameliorate OA-induced the rats' memory retrieval impairment. The prolonged latency was shortened by 15.77% (4 d, $P > 0.05$), 44.06% (5 d, $P < 0.01$) and 23.11% (6 d, $P < 0.05$) for SSFs 25 mg/kg, by 25.73% (4 d, $P > 0.05$), 54.18% (5 d, $P < 0.01$) and 34.98% (6 d, $P < 0.01$) for SSFs 50 mg/kg, by 43.78% (4 d, $P < 0.05$), 67.08% (5 d, $P < 0.01$) and 61.10% (6 d, $P < 0.01$) for SSFs 100 mg/kg, respectively. GLT 200 mg/kg can also shortened the prolonged latency by 22.05% (4 d, $P > 0.05$), 66.26% (5 d, $P < 0.01$), 56.16% (6 d, $P < 0.01$).

The effect of SSFs on OA-induced the rats' memory retention ability disturbance

Figure 5 is the result of the rats' memory retention ability on day 3 of the Morris water maze test, which was the day 31 of administration, correspond to the day 55 of operation. The results showed that intracerebroventricular injection of OA can result in the rats' memory retention ability disorder. Compared with the sham-operated group, the swimming time in the target quadrant (Q2) of the model group was shortened by 54.55% ($P < 0.01$). This shorter swimming time in Q2 was increased by 38.47% for 25 mg/kg SSFs ($P > 0.05$), 65.29% for 50 mg/kg SSFs ($P < 0.05$), 71.70% for 100 mg/kg SSFs ($P < 0.01$) and 89.55% for GLT 200 mg/kg ($P < 0.01$), respectively. The swimming trajectory distance in SSFs treated group was longer than that of model group.

Evaluation of swimming speed of rats by Morris water maze test

The rats' swimming speed was assayed on the day 7 of the Morris water maze test, which was the day 35 of administration, correspond to the day 59 of operation. The results showed that there was no significant difference in the latency of rats to find the hidden platform between each group ($P > 0.05$), which supported that these test can exclude the influences of the rats' physical strength and visual acuity in swimming behavior in Morris water maze test.

The effect of SSFs on OA-induced rats' neuropathological changes in hippocampus and cerebral cortex

Figure 6 shows the neuropathological changes of rats' hippocampus and cerebral cortex neuronal cells with HE stained. The cells layer of hippocampus marked attenuated and irregularly arranged, and accompanied by neuron loss, swollen ($\leftarrow$) and nuclear side moved ($\rightarrow$ arrow) in the model group. The neural cells in cerebral cortex showed loss, swollen (arrow $\leftarrow$), neurophagia ($\nearrow$ arrow), protrusions shorted or disappeared ($\swarrow$ arrow) in the model group. However, the abnormal changes in the neural cells induced

by OA can be ameliorated by treatment of three doses of SSFs at 25, 50 and 100 mg/kg. The neural cells were closely arranged, along with the increased cells number, the prolonged protrusions and triangular or pyramidal shape. GLT can also show the similar improvement to the abnormal neuropathological changes in the rats' brain neural cells induced by OA. Evaluation of the number of perfect neuron found that intracerebroventricular injection of OA resulted in the rats' neuron number 39.95% (hippocampus, $P < 0.01$) and 43.59% (cerebral cortex, $P < 0.01$) lower than that of sham-operated group. These decreased neuron number was increased by 15.34% (hippocampus, $P > 0.05$) and 18.61% (cerebral cortex, $P > 0.05$) for 25 mg/kg SSFs, 50.25% (hippocampus, $P < 0.01$) and 35.92% (cerebral cortex, $P < 0.05$) for 50 mg/kg SSFs ($P < 0.05$), 56.67% (hippocampus, $P < 0.01$) and 66.20% (cerebral cortex, $P < 0.01$) for 100 mg/kg SSFs ($P < 0.01$) and 25.45% (hippocampus, $P < 0.01$) and 3.71% (cerebral cortex, $P > 0.05$) for GLT 200 mg/kg, respectively.

The effect of SSFs on OA-induced ChAT protein abnormal expression in hippocampus and cerebral cortex

Figure 7 is the results of immunohistochemical detection of ChAT protein expression in the hippocampus and cerebral cortex. The pictures showed that the neurons with ChAT immunopositive expression in the sham-operated group were mostly pyramid or fusiform shape, the cytoplasm and cell membrane were yellowish brown, and the protrusions were more deeply stained. Compared with the sham-operated group, the number of ChAT protein positive expression of neurons in the model group decreased by 60.46% (hippocampus, $P < 0.01$) and 86.62% (cerebral cortex, $P < 0.01$), respectively. However, the three doses of SSFs can increase ChAT protein positive expression in the hippocampus and cerebral cortex with the increased brown-stained cells number and brown granules. The positive expression of ChAT increased by 0.24 folds (SSFs 25 mg/kg, $P < 0.05$), 0.88 folds (SSFs 50 mg/kg, $P < 0.01$) and 1.42 folds (SSFs 100 mg/kg group, $P < 0.01$) in the hippocampus, 1.82 folds (SSFs 25 mg/kg, $P < 0.01$), 4.65 folds (SSFs 50 mg/kg, $P < 0.01$) and 5.62 folds (SSFs 100 mg/kg, $P < 0.01$) in the cerebral cortex, as compared with the model group. GLT can also increased the ChAT positive expressions neuron number by 0.84 and 2.76 times in the hippocampus and cerebral cortex (all $P < 0.01$), as compared with model group.

The effects of SSFs on OA-induced hyperphosphorylation of tau at Ser262 and Ser396 in the rats' hippocampus

Figure 8 shows the results of tau-pSer262 and tau-pSer396 protein expression in the hippocampus. Compared with the sham-operated group, tau-pSer262 and tau-pSer396 increased by 84.61% ($P < 0.01$) and 74.07% ($P < 0.01$), respectively in the hippocampus of the model group. However, the three doses of SSFs reversed the above abnormal changes induced by OA, that decreased tau-pSer262 protein expression by 8.33% for SSFs 25 mg/kg, 25.00% ($P < 0.01$) for SSFs 50 mg/kg and 29.12% ($P < 0.01$) for SSFs 100 mg/kg, as well as lowered tau-pSer396 protein expression by 27.66% (SSFs 25 mg/kg, $P < 0.01$), 46.81% (SSFs 50 mg/kg, $P < 0.01$) and 70.21% (SSFs 100 mg/kg, $P < 0.01$), respectively. The GLT can also reduce the phosphorylation level of tau protein at Ser396 site by 53.19% ($P < 0.01$).

The effect of SSFs on the activities disorder of iNOS and cNOS induced by OA in rats' hippocampus and cerebral cortex

The effects of SSFs on iNOS activity disturbance induced by OA in hippocampus and cerebral cortex of rats

Figure 9 was the results of iNOS activity in the hippocampus and cerebral cortex. The data showed that the activity of iNOS increased by 1.55 times (hippocampus, $P < 0.01$) and 1.47 times (cerebral cortex, $P < 0.01$), as compared with sham-operated group. The increased activity was lowered by treatment of three doses of SSFs. The activity of iNOS decreased by 43.95% (SSFs 25 mg/kg, $P < 0.01$), 55.89% (SSFs 50 mg/kg, $P < 0.01$) and 57.47% (SSFs 100 mg/kg, $P < 0.01$) in the hippocampus, decreased by 45.63% (SSFs 25 mg/kg, $P < 0.01$), 46.27% (SSFs 50 mg/kg, $P < 0.01$) and 52.78% (SSFs 100 mg/kg, $P < 0.01$) in the cerebral cortex.

The effect of SSFs on cNOS activity disturbance induced by OA in hippocampus and cerebral cortex of rats

Figure 10 shows the results of cNOS activity in the hippocampus and cerebral cortex of rats. Compared with the sham-operated group, the cNOS activity decreased by 51.26% ($P < 0.05$) and 48.73% ($P < 0.05$), respectively, in the hippocampus and cerebral cortex of the model group. The cNOS activity increased by 1.81 times ($P < 0.01$) for SSFs 25 mg/kg, 2.4 times ($P < 0.01$) for SSFs 50 mg/kg and 2.43 times for SSFs 100 mg/kg ($P < 0.01$) in hippocampus, increased by 1.74 times ($P < 0.01$) for SSFs 25 mg/kg, 1.85 times ($P < 0.01$) for SSFs 50 mg/kg and 1.8 times ($P < 0.01$) for SSFs 100 mg/kg in the cerebral cortex. GLT can also increase cNOS activity by 1.17 times ($P < 0.01$) and 1.14 times ($P < 0.01$) in the hippocampus and cerebral cortex.

The effects of SSFs on the OA-induced the abnormal level of inflammatory cytokines IL-1 β , TNF- α and IL-6 in the rats' cerebral cortex

The effect of SSFs on IL-1 β level disorder induced by OA in the rats' cerebral cortex

Figure 11 shows the level of inflammatory factor IL-1 β in the rats' cerebral cortex. The data showed that the level of IL-1 β in the rats' cerebral cortex of the model group increased by 211.94% ($P < 0.01$), as compared with the sham-operated group. However, treatment with SSFs can reduce IL-1 β level in the cerebral cortex, that IL-1 β decreased 25.00% ($P < 0.01$) for SSFs 25 mg/kg, 37.75% ($P < 0.01$) for SSFs 50 mg/kg and 57.24% ($P < 0.01$) for SSFs 100 mg/kg in the cerebral cortex. GLT 200 mg/kg also reduced the cortical level of IL-1 β by 53.70% ($P < 0.01$).

The effect of SSFs on TNF- α level disorder induced by OA in the rats' cerebral cortex

The effects of SSFs on TNF- α level in cerebral cortex induced by OA

Figure 12 shows the level of inflammatory factor TNF- α in the rats' cerebral cortex. The results showed that the level of TNF- α in the rats' cerebral cortex of the model group increased by 211.94% ($P < 0.01$), as compared with the sham-operated group. However, treatment with SSFs can reduce TNF- α level in the cerebral cortex, that TNF- α decreased 2.61% ($P > 0.05$) for SSFs 25 mg/kg, 9.17% ($P > 0.05$) for SSFs 50 mg/kg and 14.45% ($P < 0.05$) for SSFs 100 mg/kg in the cerebral cortex. GLT 200 mg/kg also reduced the cortical level of TNF- α by 44.81%, ($P < 0.05$).

The effect of SSFs on IL-6 level disorder induced by OA in the rats' cerebral cortex

Figure 13 shows the level of inflammatory factor IL-6 in the rats' cerebral cortex. The results showed that the level of IL-6 in the rats' cerebral cortex of the model group rose by 32.95% ($P < 0.05$), as compared with the sham-operated group. However, treatment with SSFs can lower IL-6 level in the cerebral cortex, that IL-1 β reduced 28.38% ($P > 0.05$) for SSFs 25 mg/kg, 44.64% ($P < 0.05$) for SSFs 50 mg/kg and 73.72% ($P < 0.01$) for SSFs 100 mg/kg in the cerebral cortex. GLT 200 mg/kg also attenuated the cortical level of IL-6 by 24.64%, but no significant difference ($P > 0.05$).

Discussion

AD is a progressive neurodegenerative disease that seriously threatens the physical and mental health of the elderly. The disease is pathologically associated with A β plaque deposition, tau hyperphosphorylation, cholinergic damage, neuroinflammation and oxidative stress [11, 12]. OA, as an inhibitor of protein phosphatases PP1 and PP2A, can reduce the dephosphorylation of phosphorylated tau protein and result in the tau protein hyperphosphorylation, which in turn produces NFT deposition and indirectly develops AD [13]. Then, the brain injured AD model established by intracerebroventricular injection of OA in rats is consistent to the pathogenesis of AD patients.

Generally speaking, the memory is composed of three parts of memory acquisition, retention, and retrieval. The hippocampus and cerebral cortex are the key regions of learning and memory formation and storage, and the integrity of their structure directly affects learning and memory function [14]. Neuronal loss and neuropathological changes occur in AD patients' brains, especially the loss in hippocampus. However, SSFs can significantly improve the rats' learning and memory impairment and neuropathological changes induced by OA, and are parallel to the protection of neuronal pathological changes, which indicated that the improvement of SSFs in memory impairment is related to its neuro-protection.

It is well known that tau protein is a neuronal protein that regulates microtubule structure and cell integrity. It is an essential molecule for assembling and stabilizing the microtubule cytoskeleton [15]. Many studies have shown that the phosphorylation of tau protein at Ser 262 and Ser 396 sites may fail to form stable microtubules and then result in the normal axonal transport and microtubule dysfunction, and neurodegeneration [16–18]. In the present study, the intracerebroventricular injection of OA to rats can significantly increase tau protein hyperphosphorylation at Ser262 and Ser396 sites in the brain and SSFs can reverse the above changes, which agreed with the amelioration in the memory impairment and

neuropathological changes of SSFs. These results provided evidence that SSFs improve the memory impairment and neuronal damage may be derived from the inhibition in tau hyperphosphorylation.

There is confluence of opinion that acetylcholine (ACh) is a key neurotransmitter in regulating the neural cognition. The level of ACh in the neuron was mainly managed by ChAT. The decreased ChAT activity in AD is primary from the hyperphosphorylated tau protein neurotoxicity. The hyperphosphorylated tau protein attacks the neural structure and prevents ChAT and ACh to transport to the hippocampus and cerebral cortex and finally destroys the neural function [19–20]. The present studies have showed that the intracerebroventricular injection of OA can significantly lower the protein expression of ChAT in the brain and the decreased protein expression of ChAT was markedly reversed by SSFs treated. This melioration of SSFs in ChAT disorder was consistent with the improvement in memory impairment, neuropathological changes and tau hyperphosphorylation induced by OA. These results suggested that the improvement of SSFs in memory impairment and neuropathological changes induced by OA might be primary from enhancing ChAT activity of the brain.

There has been evidence showing that NO, an important neurotransmitter in the central nervous system, plays an important role in cognitive function. NOS is the rate-limiting enzyme of NO synthesis, which was divided into inducible type (iNOS) and structural type (cNOS) [21]. iNOS, mainly presented in the macrophages, is Ca^{2+} -dependent enzyme on the initiating stage and catalyze to produce abundant of NO. cNOS, mainly existed in the endothelial and neural cells, is Ca^{2+} and calmodulin-dependent enzyme, and catalyze to product a small amount of NO [22]. Then, only when the activity of the two NOS is at equilibrium, the normal function of NOS can be guaranteed. In AD patients, the high level $\text{A}\beta$ in neuron can cause a large amount of Ca^{2+} influx and intracellular Ca^{2+} overload, which prompts iNOS high expression and cNOS decreased expression and advance to produce an amount of NO. The excessive NO can be regarded as free radical ONOO^- to inhibit the phosphorylated tau protein dephosphorylation to produce high NFTs aggregation [23, 24]. In this study, we found that OA significantly decreased cNOS and increased iNOS activity in brain. SSFs can significantly reverse the increased iNOS activity and the decreased cNOS activity induced by OA. This effect of SSFs is due to the fact of flavonoids with polyhydroxy structure, which exerts a strong reducibility and antioxidant activity. The antioxidant activity of SSFs has the strong protection for biomembrane, lowers the cell membrane permeability and Ca^{2+} influx and calcium overload. This result is from the down-regulating iNOS and up-regulating cNOS activities, and attenuate free radical ONOO^- production, neural damage and memory impairment of SSFs. The regulations of SSFs in iNOS and cNOS agreed to its neuroprotection and memory impairment improvement, which suggests that the anti-AD activity of SSFs is associated to its positive regulation in iNOS and cNOS activity.

There is mounting evidence to suggest that many cytokines contribute to the neural genesis, development and injury repairmen. IL-1 β , TNF- α and IL-6 will become the inflammatory cytokines when the brain is attacked by some detriment [25, 26]. Under the physiological condition, IL-1 β promotes neuronal growth and development, whereas, under the pathological conditions, IL-1 β not only prompts the cytokines

synthesis and release of TNF- α and IL-6 but also promotes tau hyperphosphorylation and aggravates the cognitive dysfunction [27]. TNF- α mainly secreted from macrophages, accumulates around of glial cell and promotes the deposition of A β in the brain [28]. IL-6 has dual function that can induce neurodegeneration and cell death, and also inhibit the deposition of A β in vivo [29]. The present studies have found that the intracerebroventricular injection of OA to rats significantly increased the levels of IL-1 β and TNF- α , and decreased the level of IL-6 in the cerebral cortex. However, SSFs can lower IL-1 β , TNF- α and enhance IL-6 levels resulted from OA, which indicated that SSFs showed an anti-neuronal inflammatory activity. The present study results showed that the regulation of SSFs in inflammatory cytokines is confluence to its improvement in neuropathologies and memory impairment caused by OA, which confirmed that the amelioration of SSFs on OA-induced memory impairment is concerned with the regulation of IL-1 β , TNF- α , and IL-6 levels.

The present study also found that the positive control drug GLT has certain positive regulation on OA-induced the rats' memory impairment, neuropathological changes, ChAT, tau-pSer262, tau-pSer396, NOS and IL-1 β , TNF- α and IL-6 abnormal changes, and the effect of GLT is parallel to SSFs. Both GLT and SSFs are belonged to flavonoids, which contain polyhydroxy structures and have strong reducibility. They play a neuroprotective role by protecting bio-membrane and improving memory impairment for anti-AD, which indicated that flavonoids may be beneficial in the treatment of AD.

Conclusion

In summary, SSFs markedly improve OA-induced the rats' learning and memory impairment and neuropathological changes. This effective mechanism of SSFs stem from the positive regulation in ChAT activity increased, tau protein hyperphosphorylation reduced, NOS activity regulated and inflammation resisted. The present study, combined with our previous reports, suggested that SSFs may be hopeful strategy for treatment of AD.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Dr. Shang Yazhen and Li Caixia conceived and designed the study. Ma Shuai, Xu Congcong and Dong Yongcai performed the experiments and analyzed the data. Ma Shuai and Xu Congcong drafted the manuscript and Shang Yazhen revised the manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

All procedures for all animals in the study were approved by the Animal Ethics Committee of Chengde Medical College (No. CDMU-LAC-20180313013).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures



Original plant



Dry herb



SSFs extract

Figure 1

Pictures of *Scutellaria baicalensis* Georgi original plant, dry herb and SSFs extract.

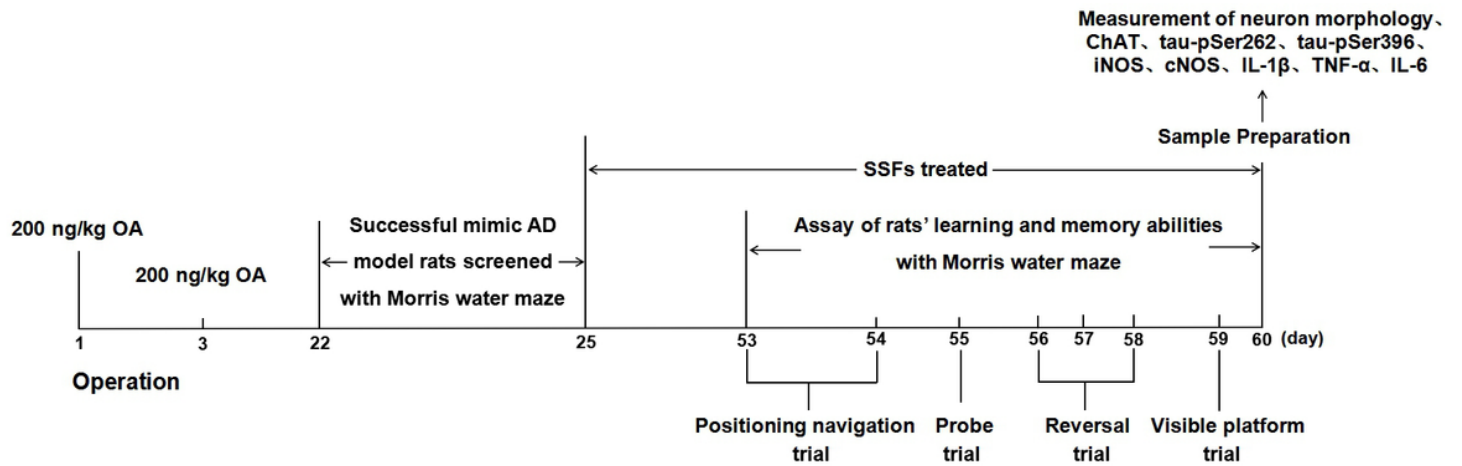


Figure 2

The timeline of the experiment designed

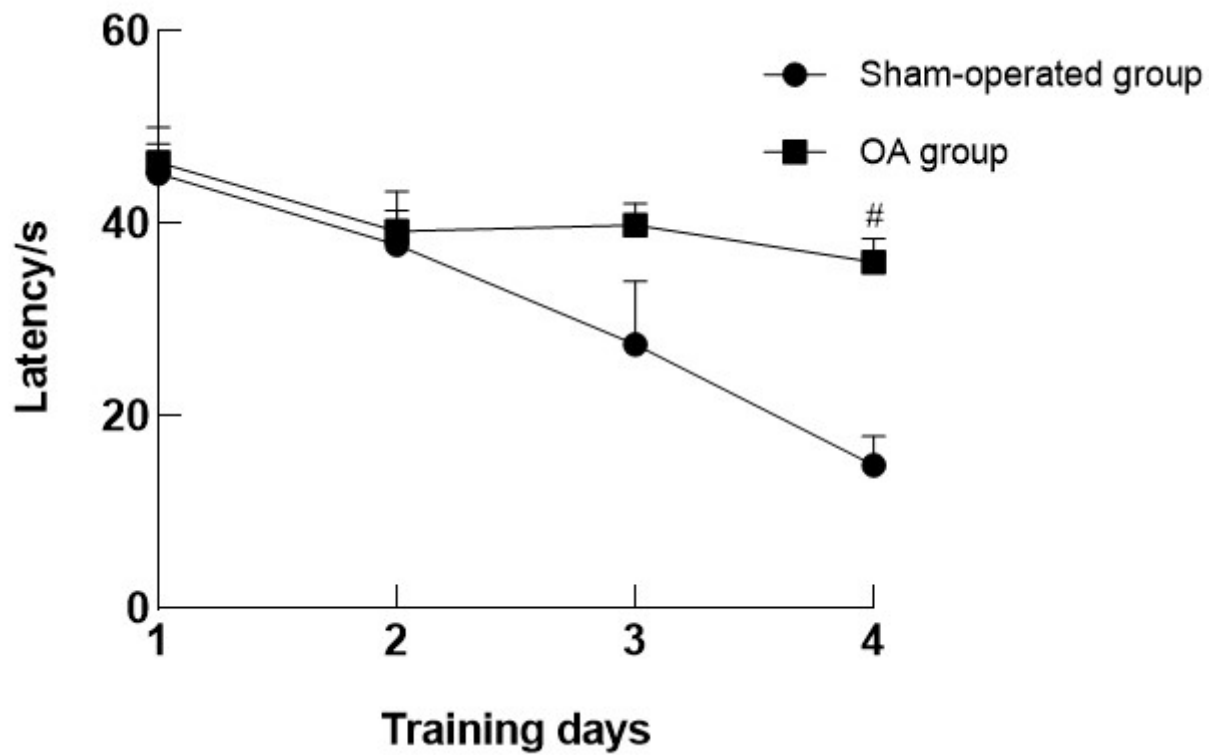


Figure 3

The successful mimic AD model rats screened with Morris water maze training. The SR was calculated from the results of rats' training on the day 4 with Morris water maze. Data were as mean $\pm$ SD. [#] $P < 0.05$, vs sham-operated group.

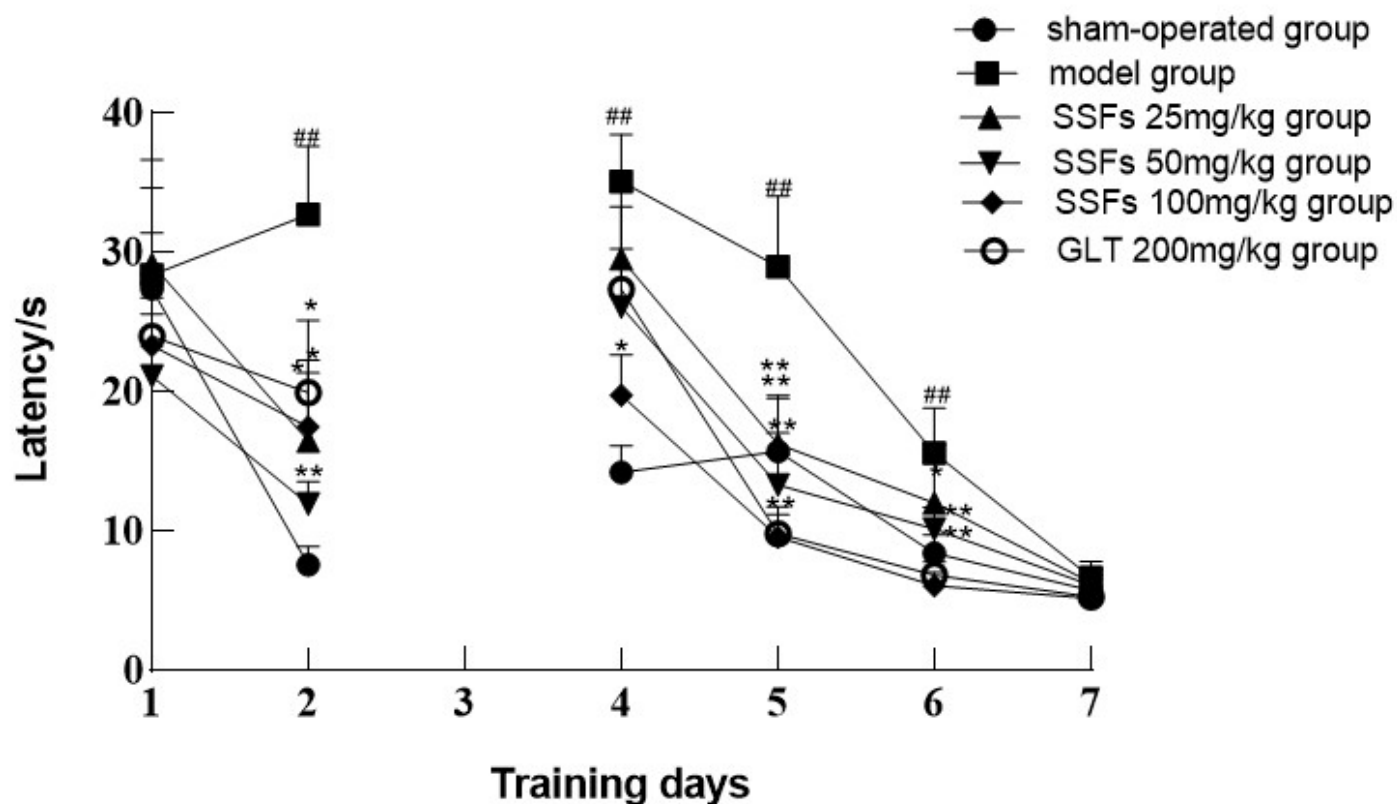


Figure 4

The effect of SSFs on OA-induced the rats' learning and memory acquisition ability and retrieval ability impairment. The positioning navigation trial was performed on day from 29 to 30 of treatment with SSFs, corresponded to from day 53 to 54 after the operation for testing the rats' learning and memory acquisition ability. The reversal trial was carried out from day 32 to 34 of treatment with SSFs, corresponded to from day 56 to 58 after the operation for testing the rats' retrieval ability. Data were analyzed by two-way ANOVA (day×group) with repeated measures. Mean $\pm$ SD, ## P <0.01, vs sham-operated group, ** P <0.05, * P <0.01, vs model group.

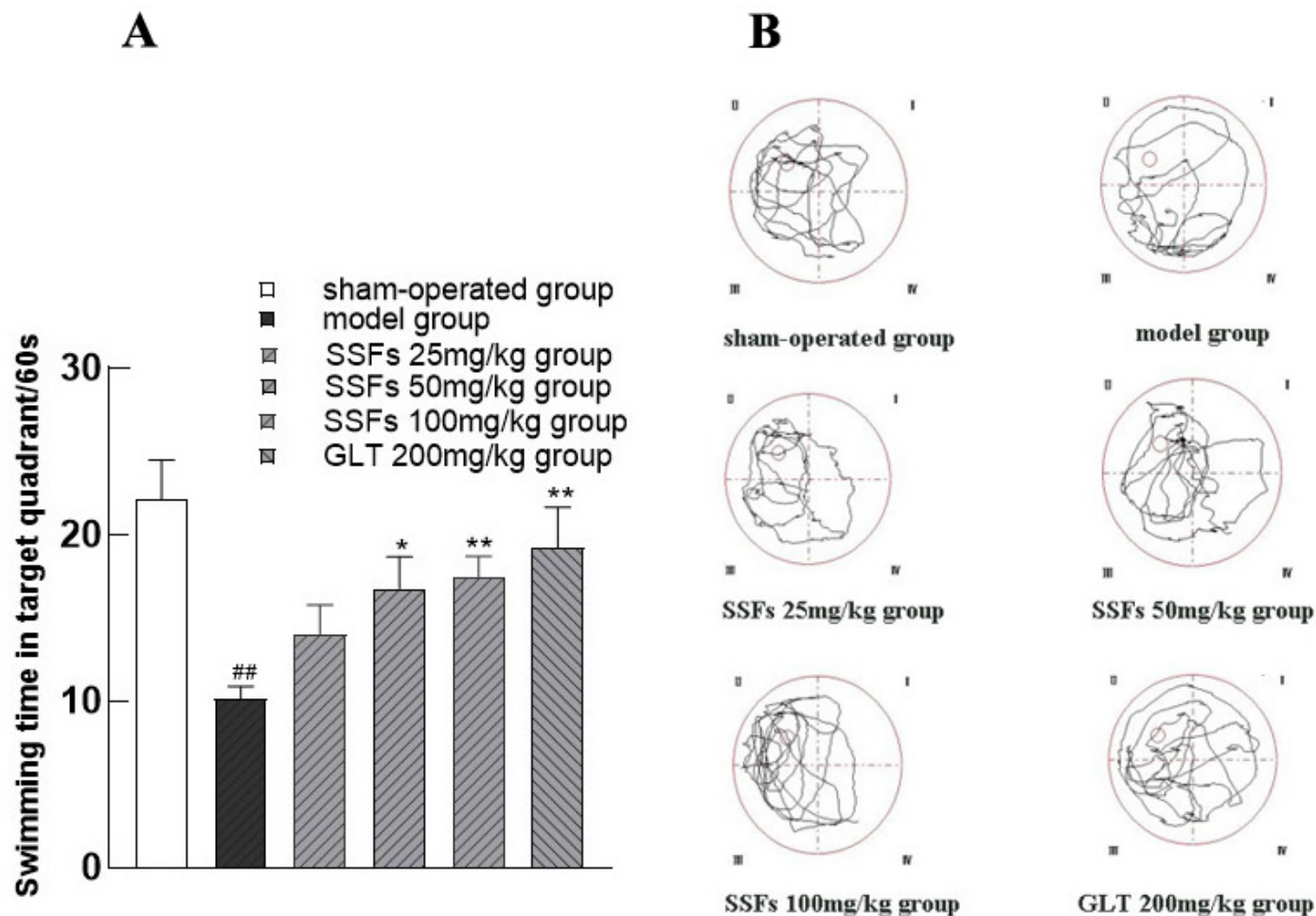


Figure 5

Effects of SSFs on the rats' memory retention ability impairment induced by OA. The probe trial was performed on day 31 of treatment with SSFs, corresponded to 55 of the operation for testing the rats' memory retention ability. A: The swimming time of rats in the target quadrant within 60 s in the probe trial (no platform). B: The swimming trajectory of rats in probe trial. BA: sham-operated group, BB: model group, BC: SSF 25 mg/kg group, BD: SSF 50 mg/kg group, BE: SSF 100 mg/kg group, BF: GLT 200 mg/kg group. Mean $\pm$ SD. ## P <0.01, vs sham-operated group. * P <0.05, ** P < 0.01, vs model group.

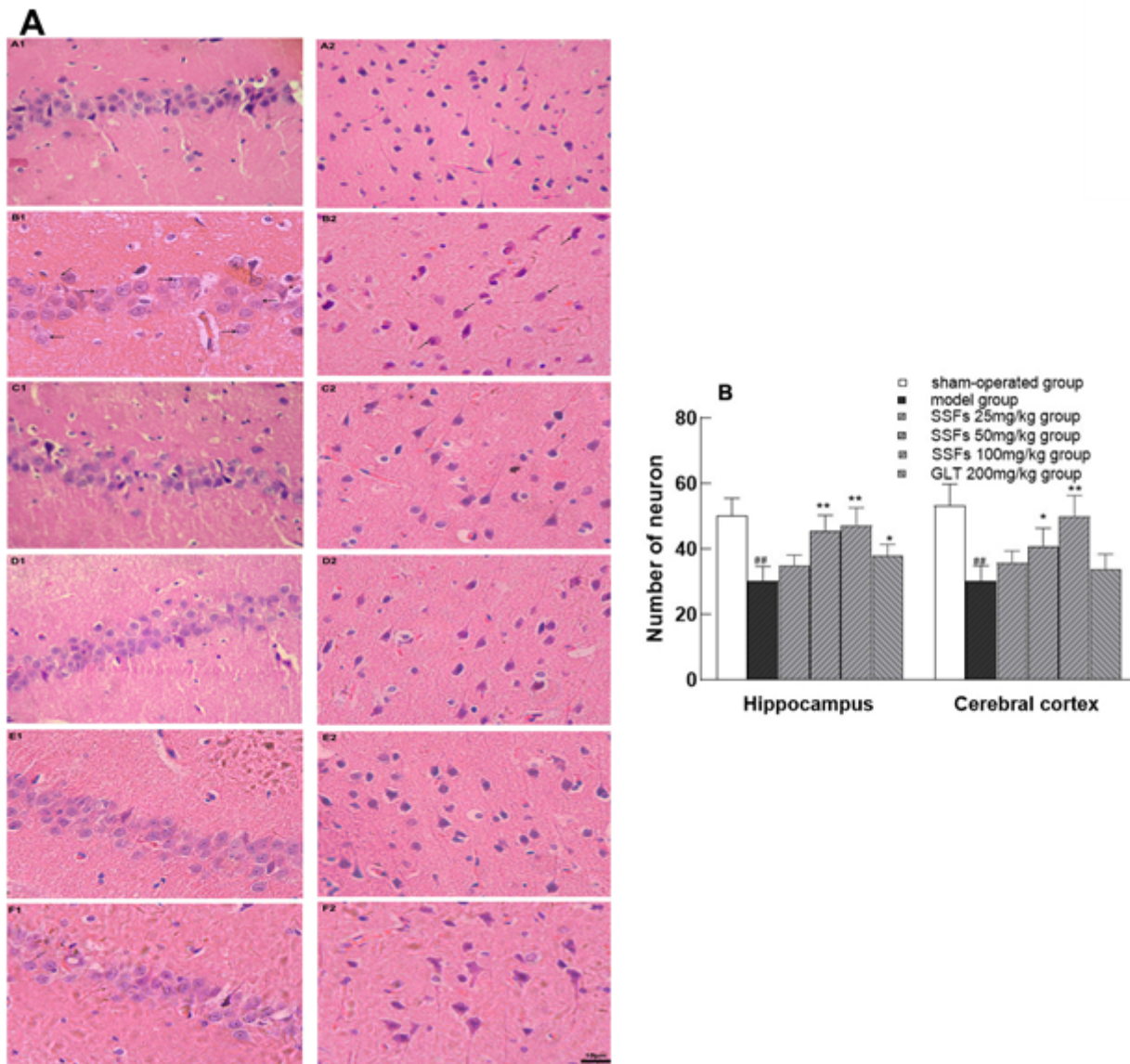


Figure 6

A Representative photomicrographs of neuron in hippocampus and cerebral cortex. A1-A2: sham-operated group; B1-B2: model group, showing neuron loss and swelling ($\leftarrow$ arrow), neuronophagia ($\nearrow$ arrow) and nuclear side moved ($\rightarrow$ arrow), protrusions shorted or disappeared ($\swarrow$ arrow). C1-C2: 25mg/kg SSFs group; (D1-D2) 50mg/kg SSFs group; (E1-E2): 100mg/kg SSFs group, (F1-F2): 200mg/kg GLT group. Three doses of SSFs and GLT showed notable protection against OA-induced neuronal pathological changes. (A1-F1) hippocampus, $\times 400$ magnification. (A2-F2) cerebral cortex, $\times 400$ magnification. HE staining (400 $\times$), Scale bar: 10 μ m. **B** Effects of SSFs on rats' neuron number in hippocampus and cerebral cortex, which was counted under the light microscope (400 $\times$). Each volume represents mean $\pm$ SD from 9 visible areas of 3 independent samples (n=3) and analyzed by One-way ANOVA. ^{##} $P < 0.01$, vs sham-operated group. ^{*} $P < 0.05$, ^{**} $P < 0.01$, vs model group.

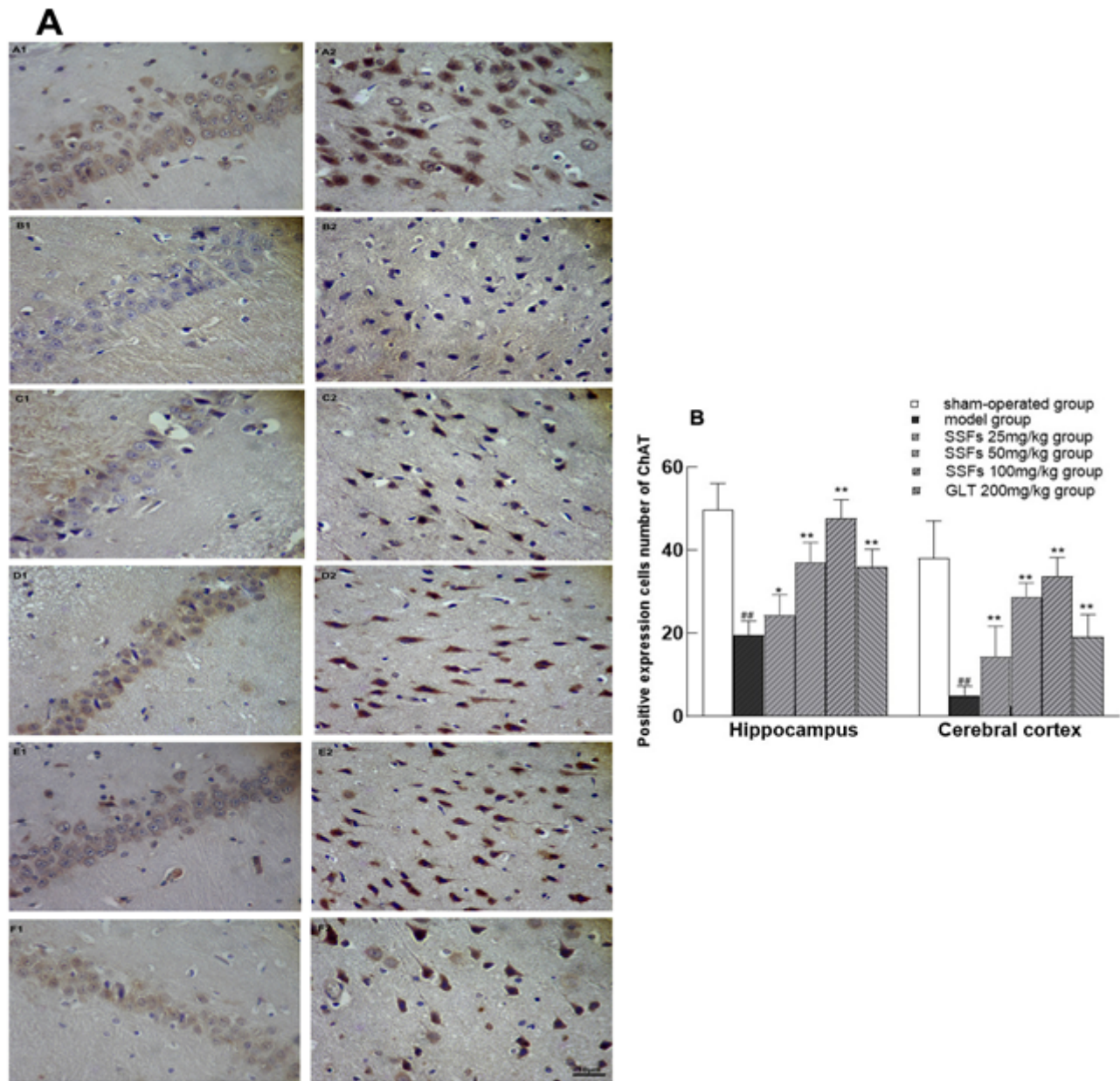


Figure 7

A Representative photomicrographs of neuron ChAT immunohistochemistry assayed in hippocampus and cerebral cortex. A1-A2: sham-operated group; B1-B2: model group, showing neuron light yellowish brown. C1-C2: 25mg/kg SSFs group; (D1-D2) 50 mg/kg SSFs group; (E1-E2): 100 mg/kg SSFs group, (F1-F2): 200 mg/kg GLT group. Three doses of SSFs and GLT showed notable protection against OA-induced the decreased ChAT protein expression. (A1-F1) hippocampus, $\times 400$ magnification. (A2-F2) cerebral cortex, $\times 400$ magnification. Immunohistochemistry (400 $\times$), Scale bar: 10 μ m. **B** Effects of SSFs on the number of rats' ChAT immunohistochemistry stained in hippocampus and cerebral cortex, which was counted under the light microscope (400 $\times$). Each volume represents mean $\pm$ SD from 9 visual regions of 3 independent samples ($n=3$) and analyzed by One-way ANOVA. ## $P<0.01$, vs sham-operated group. * $P<0.05$, vs model group.

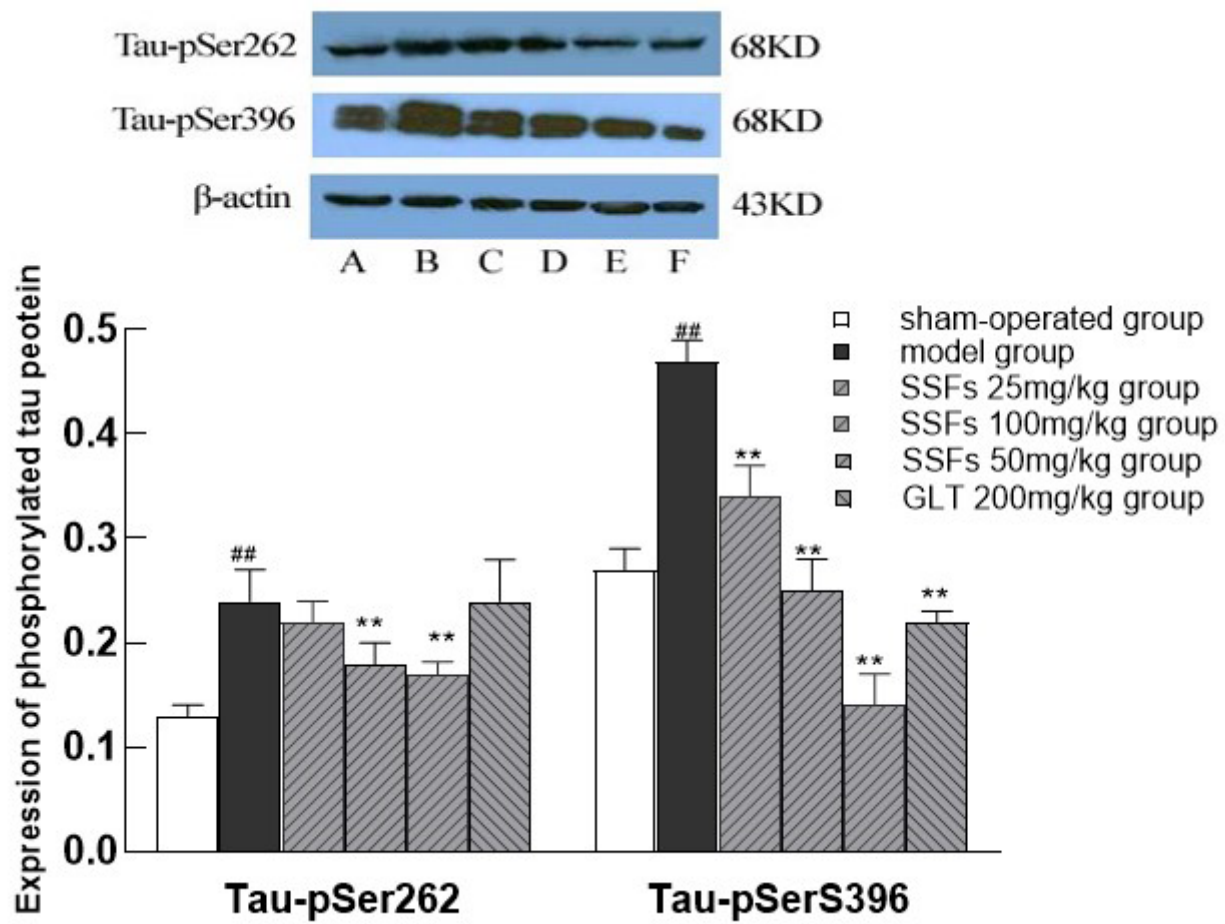


Figure 8

Effects of SSFs on hyperphosphorylation of tau at Ser262 and Ser396 induced by OA in rat hippocampus. Every column represents the expressive level of tau-pSer262 and tau-pSer396 protein. Data were analyzed by One-way ANOVA (mean±SD). ^{##} $P < 0.01$, vs the sham-operated group. ^{**} $P < 0.01$, vs the model group.

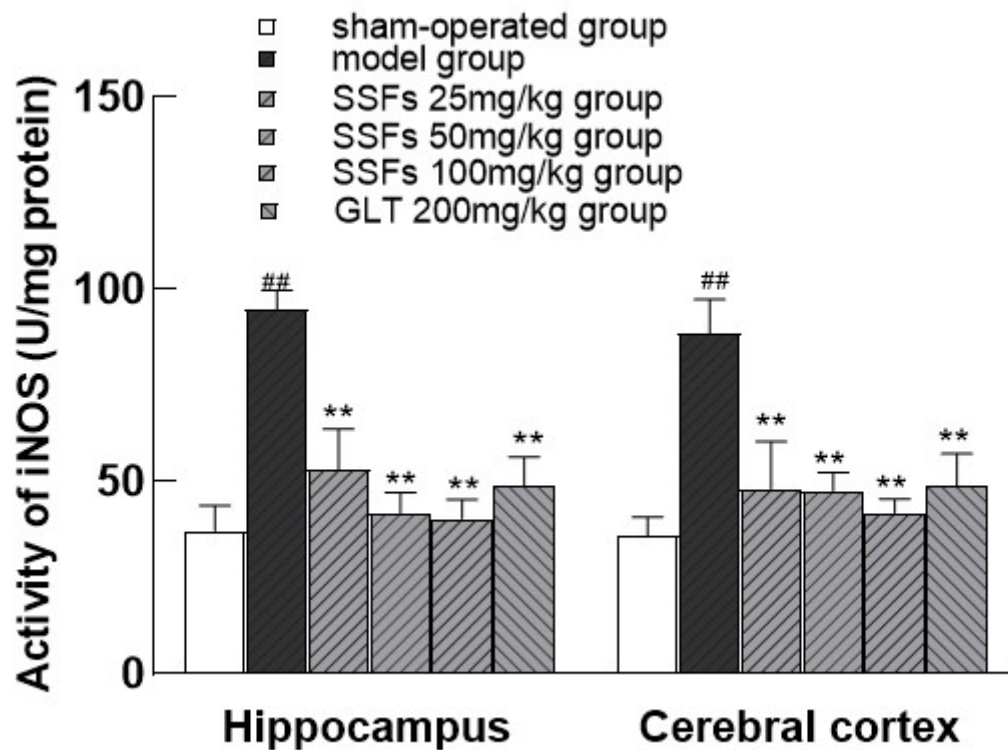


Figure 9

Effect of SSFs on iNOS activity disorder induced by OA in hippocampus and cerebral cortex of rats. Every column represents the average activity of iNOS. Data were analyzed by One-way ANOVA (mean $\pm$ SD).

^{##} $P < 0.01$, vs sham-operated group. ^{**} $P < 0.01$, vs model group.

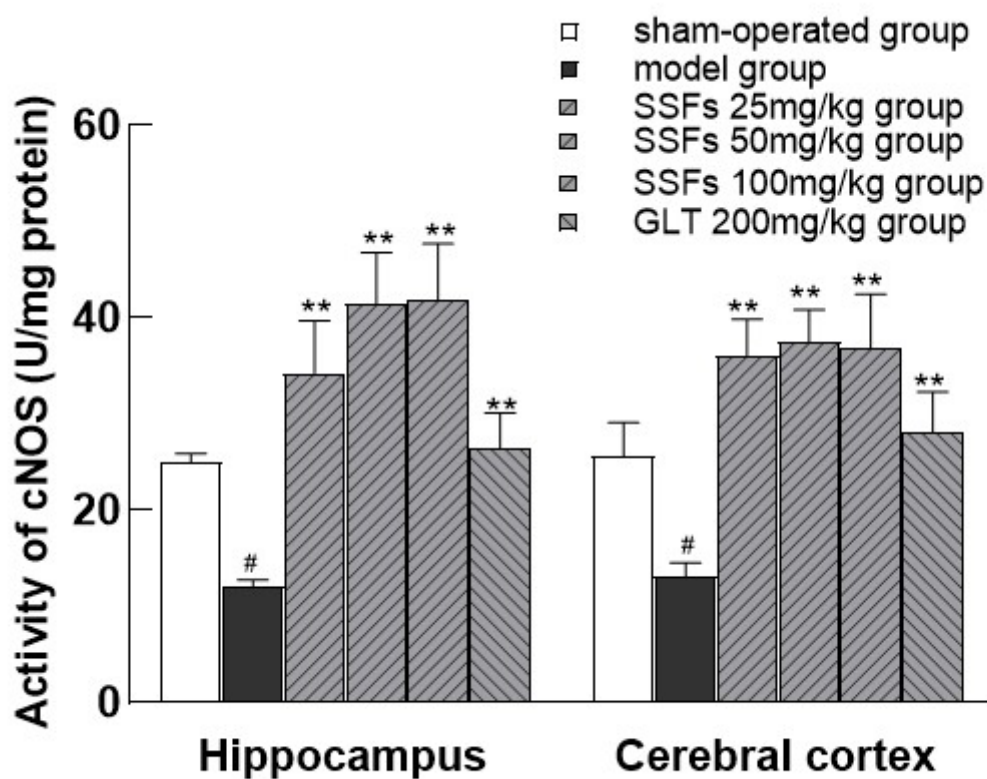


Figure 10

The effects of SSFs on cNOS activity induced by OA in hippocampus and cerebral cortex of rats. Every column represents the average activity of cNOS and analyzed by One-way ANOVA (mean $\pm$ SD). [#] $P < 0.05$, vs sham-operated group. ^{**} $P < 0.01$, vs model group.

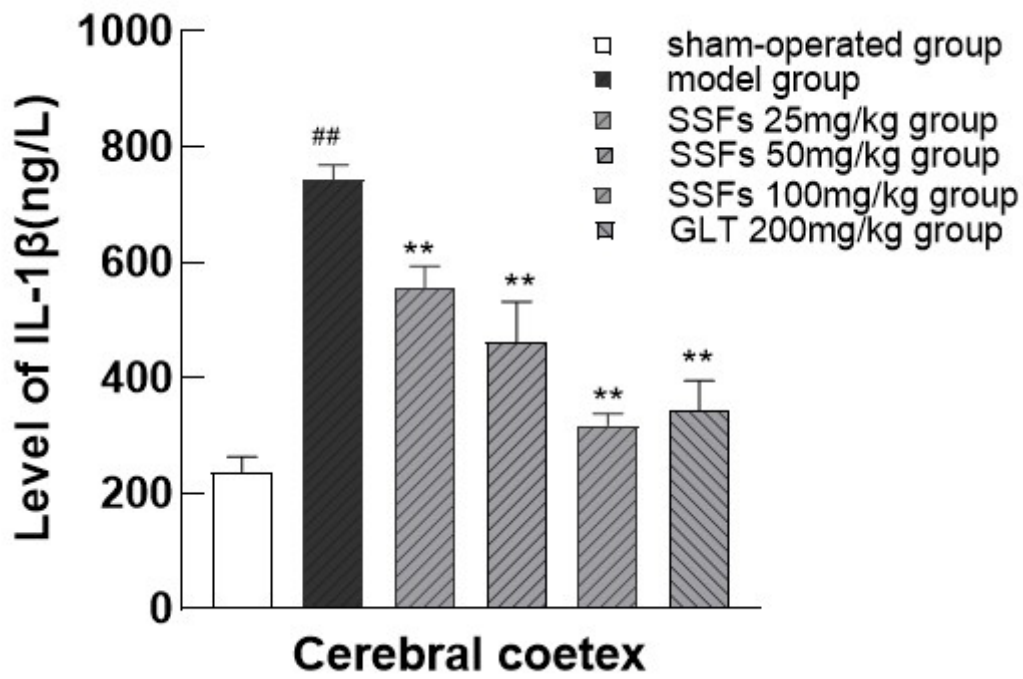


Figure 11

Effect of SSFs on IL-1 β level in rat cerebral cortex induced by OA. Every column represents the average level of IL-1 β . Data were analyzed by One-way ANOVA (mean $\pm$ SD). ## P <0.01, vs sham-operated group. ** P <0.01, vs model group.

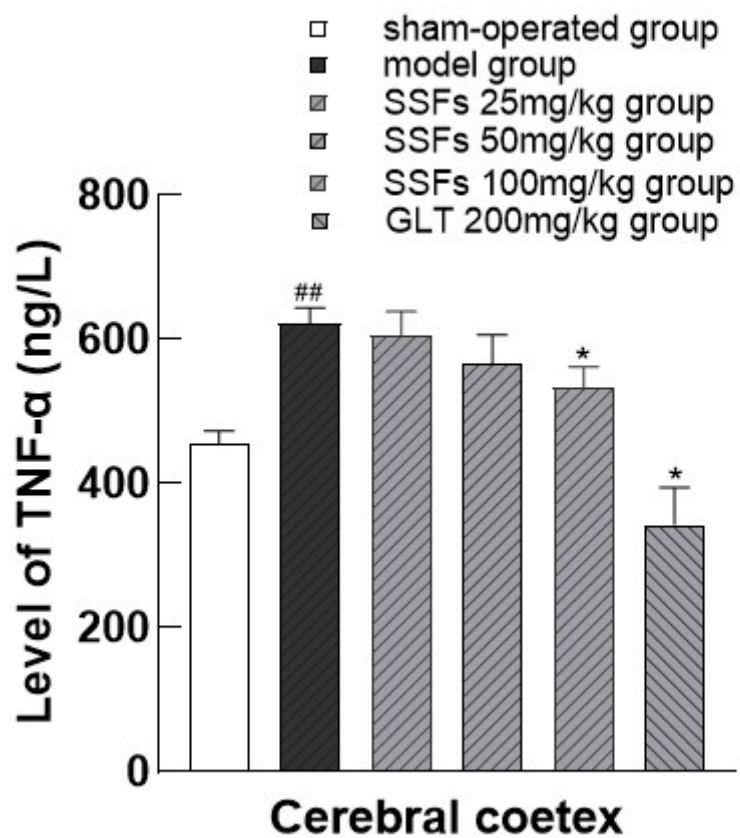


Figure 12

Effect of SSFs on TNF-α level in rat cerebral cortex induced by OA. Every column represents the average level of IL-1β. Data were analyzed by One-way ANOVA (mean ± SD). ## $P < 0.01$, vs sham-operated group. * $P < 0.01$, vs model group.

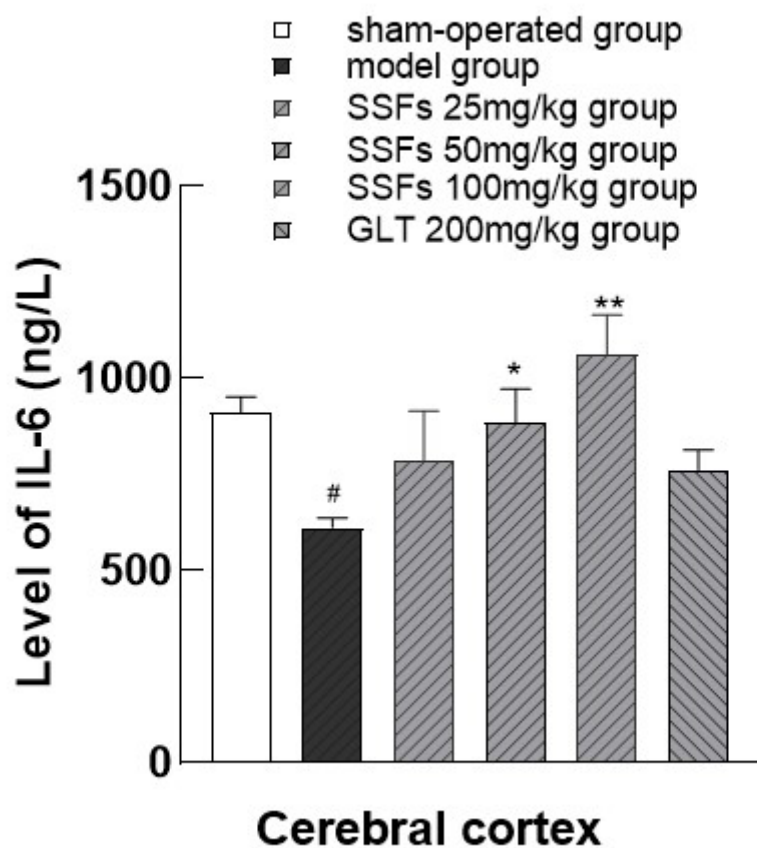


Figure 13

Effect of SSFs on IL-6 level in rat cerebral cortex induced by OA. Every column represents the average level of IL-1 β . Data were analyzed by One-way ANOVA (mean $\pm$ SD). # P <0.01, vs sham-operated group. * P < 0.05, ** P <0.01, vs model group.