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Neuroprotective Effect of Astragalus Polysacharin on Streptozotocin (STZ)-Induced Diabetic Rats

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: In the recent years, there has been increasing interest in traditional Chinese medicine as a neuroprotective nutrient in the management of chronic neurodegenerative disease, such as diabetic cognitive decline. Astragalus polysacharin (APS), a Chinese herb extract, is a biologically active treatment for neurodegenerative diseases. Therefore, in the present study, we investigated the neuroprotective effects of APS (20 mg/kg) on diabetes-induced memory impairments in Sprague-Dawley (SD) rats and explored its underlying mechanisms of action.

Material/Methods: Thirty SD rats were randomly divided into a control group (CON group, n=10), a diabetic model (DM) group (n=10), and an APS group (n=10). We administered 55 mg/kg streptozotocin (STZ, Sigma) by intraperitoneal injection to induce a diabetic model. Food and water intake, body weight, and blood fasting plasma glucose (FPG) were measured. The Morris water maze test (MWM) was used to assess learning and memory ability, and we measured levels of N-methyl-D-aspartate receptor (NMDA), calcium/calmodulin-dependent protein kinase II (CaMKII), and cAMP response element-binding protein (CREB) in the hippocampus.

Results: APS (20 mg/kg) administration decreased the rats' fasting plasma glucose (FPG) levels and body weight. APS (20 mg/kg) administration improved the cognitive performance of diabetes-induced rats in the Morris water maze test. APS (20 mg/kg) administration reduced the number of dead cells in the CA1 region of the hippocampus. Furthermore, APS (20 mg/kg) administration obviously upregulated the phosphorylation levels CREB, NMDA, and CaMK II.

Conclusions: These results suggest that APS has the neuroprotective effects, and it may be a candidate for treatment of neurodegenerative diseases such as diabetic cognitive impairment.

MeSH Keywords: **Anti-N-Methyl-D-Aspartate Receptor Encephalitis • *Astragalus Membranaceus* • CREB-Binding Protein • Diabetes Complications • Mild Cognitive Impairment**

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Background

Diabetes is a highly prevalent endocrine disease with incidence rapidly increasing every year [1]. Diabetes mellitus (DM) can cause complications in multiple organs, such as the heart, eyes, lower-limb blood vessels, lungs, and brain. Increasing evidence in diabetic animal models also showed that diabetes induces cognitive impairment and memory loss. Diabetes is an important risk factor for cognitive dysfunction, but the exact mechanism is unclear. The hippocampus is an important brain area of learning and memory ability, and it is reported that sustained hyperglycemia can lead to hippocampal ultrastructural damage [2,3].

Increasing evidence demonstrates that neurological diseases involved in diabetes are related to synaptic plasticity [4]. Incomplete synapses can lead to blocked neurosignaling. Possible mechanisms are related to synaptic dysplasia. NMDA, CaMK II, and CREB are the neural plasticity-related proteins, and the level of their expression can affect synaptic formation and plasticity, as well as learning and memory.

Astragalus polysacharin (APS) is a main bioactive ingredient of the plant genus Astragalus, which has a variety of pharmacological and physiological functions, including immunomodulatory effect [5–8], anti-cancer action [9–11], liver protection [12], and treatment of diabetes [13]. It is reported that APS has a good therapeutic effect on diabetes, lowering blood glucose and body weight. APS was identified as a candidate agent to reverse cognitive dysfunction [14]. In this study, we explored whether APS can prevent memory disfunction in a STZ-induced diabetic model and assessed its underlying mechanisms.

Material and Methods

Experimental animals

Thirty male 13-weeks-old Sprague-Dawley (SD) rats were obtained from the Laboratory Animal Center of Tianjin Medical University (Tianjin, China), and housed in a thermostatically controlled room (22±1°C) with a 12 h light/dark cycle (light on 7 a.m.–7 p.m.), and with free access to food and water. The experiment was approved by of Ethics Committee of the Tianjin Medical University. The rats were randomly divided into 3 groups: control group (n=10), DM group (n=10) and APS group(n=10). Except for control rats, the other rats received intraperitoneal injection with 55mg/kg STZ. Diabetic rat model was induced by STZ (55 mg/kg), which was based on previous research [15]. The DM rats were determined by fasting blood glucose ≥16.7 mmol/L 72 h after STZ injection. The rats in APS model group were treated with APS (20 mg/kg) intragastrically once daily for consecutive 10 weeks. After treatment, the 24-hour food and water intakes, body weight, FPG, biochemical experiments and behavioral tests were performed in sequence.

Laboratory testing

FPG was measured every week. At 24 h after the last drug treatment, venous blood was collected for FPG measurements. Body weight and water intake of rats were measured dynamically for 10 weeks after the STZ injection.

Water Morris maze

The Morris water maze (MWM) [16] had a diameter of 150 cm, height of 50 cm, water depth of 40 cm, and temperature of 22±1°C. A hidden platform (10 cm in diameter) was submerged 1 cm below the surface of the water and placed in the middle of the same quadrant throughout the training phase. Over the next 4 days (1–4 d), the rats (n=10 per group) underwent 3 consecutive trials per day with intervals of 5 min. In each trial, an individual rat was placed into the water facing the pool wall and permitted to search for the submerged platform for 120 s. If a rat did not locate the platform within 120 s, it would be gently placed on the platform for 20 s, and the escape latency was recorded as 120 s. The mean escape latency of 3 trials was noted as the daily result of learning ability for the animal. On the 5th day of the test, each rat was subjected to a probe trial session in which the platform was removed from the pool, and the rats were allowed to swim for 120 s to search for it. The frequency with which each rat passed the hidden platform and the resident time that each rat spent in the target quadrant were noted as the result of the spatial memory function.

Hematoxylin-Eosin (HE) staining

After WMW testing, the rats were anesthetized with 10% chloral hydrate (4 ml/kg) i.p. SD rats were sacrificed and the brain tissue was rapidly stripped and postfixed for 24 h in formalin. After postfixing, tissues were embedded in paraffin wax and 5-μm-thick serial coronal sections were obtained and mounted on polylysine-coated glass slides. For histological assessment of damage to the hippocampus, the paraffin-embedded tissues were stained with hematoxylin-eosin (HE) according to standard protocol.

Western blot

Rats were sacrificed under deep anesthesia (2.5 g/kg urethane). For Western blot analysis, the rats were perfused transcardially with 0.9% saline (PBS; pH 7.2–7.4). The rat hippocampus tissue was isolated, then placed in liquid nitrogen. Sample preparation and protein determination were performed. Tissues

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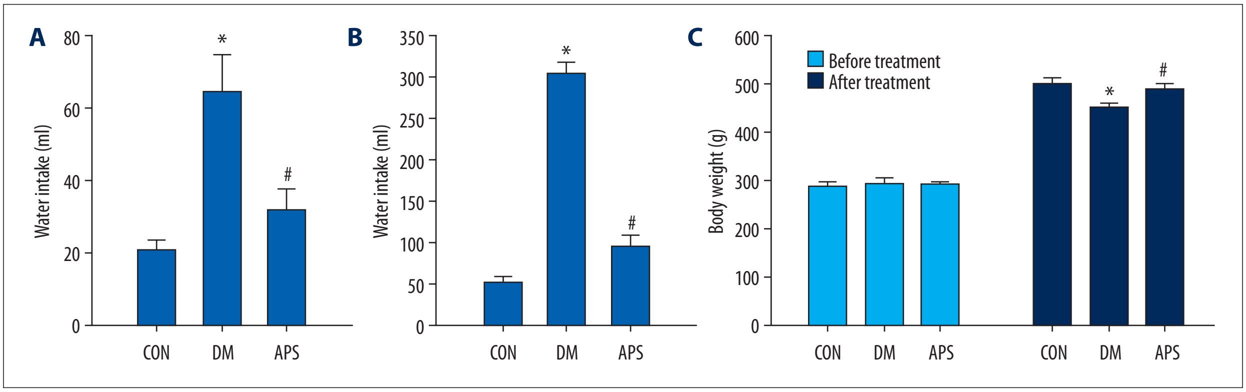

Figure 1. Food and water intake and body weight were measured (**A–C**). Statistical analysis was by repeated-measures ANOVA followed by least significant difference (LSD) post hoc analysis. * p<0.01 *vs.* CON group, # p<0.01 *vs.* DM group.

samples were digested with RIPA lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Nonidet- 40, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 1 mmol/L PMSF) with protease inhibitors (pepstatin 1 μg/mL, aprotinin 1 μg/mL, leupeptin 1 μg/mL) for 30 min, and the protein concentration was determined using the BCA assay kit (Abcam, ab207003, UK). Different samples with an equal amount of protein were separated using 8–12% SDS-polyacrylamide gels and transferred to PVDF membranes. After blocking with 10% non-fat milk at room temperature, the membranes were incubated with primary antibodies against Anti-NMDAR1 (phospho S897) (1: 500; Abcam, ab207003, UK), Anti-CaMKII (phospho T286) (1: 500; Abcam, ab171095, UK), Anti-CREB (phospho S133) (1: 500; Abcam, ab32096, UK), and β-actin (1: 500; Abcam, ab49900, UK) at 4°C overnight. After rinsing, the membranes were appropriately incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1: 5000; Abcam, ab6785, UK) for 2 h at room temperature.

Statistical analysis

Statistical analysis was performed in SPSS 17.0. All data are presented as mean ±SD. Differences among 3 or more groups were compared by one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) post hoc analysis. p values of 0.05 or less were regarded as significant.

Results

APS influenced body weight and water and food intake in diabetic rats. Diabetic rats had significantly higher food and water intake (*p*<0.01). After APS treatment, the water and food consumption of rats with diabetes decreased significantly (Figure 1A, 1B). The weight of rats in the DM group decreased obviously (*p*<0.01), whereas APS administration reversed the body weight changes in STZ-induced diabetic rats (Figure 1C).

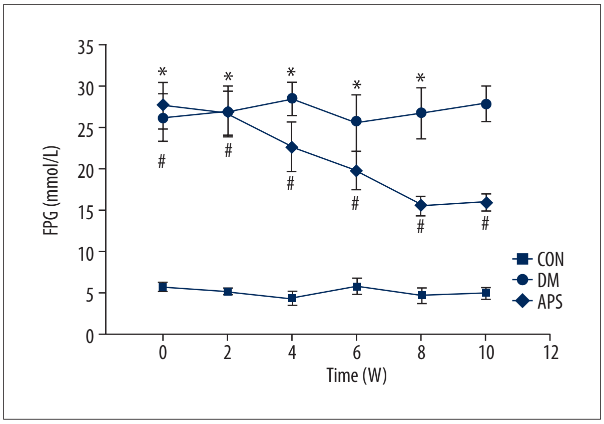

Figure 2. FPG levels of different groups at different time points. Statistical analysis was by repeated-measures ANOVA followed by least significant difference (LSD) post hoc analysis. * p<0.01 *vs.* CON group, # p<0.01 *vs.* DM group.

APS decreased FPG levels in diabetes rats, as revealed by dynamic testing of all experimental animals with fasting blood glucose for 10 weeks. Compared with the control group, the blood glucose of the diabetic group was significantly higher (p<0.01). After administration of APS for 10 weeks, rat blood glucose levels were significantly lower than in the DM group (p<0.01) (Figure 2).

Regarding effects of APS on cognitive deficit in STZ-induced diabetic rats, APS significantly improved the learning and memory ability of diabetic rats. Compared with the CON group, escape latency of diabetic rats was significantly shorter (p<0.01). However, APS restored the escape latency (p<0.01 *vs.* DM group) (Figure 3A). In the probe test, the platform crossings of the target quadrant of diabetic rats was significantly shorter than that of the CON group (p<0.01), whereas APS treatment reversed the platform crossing performance in diabetic rats (Figure 3B).

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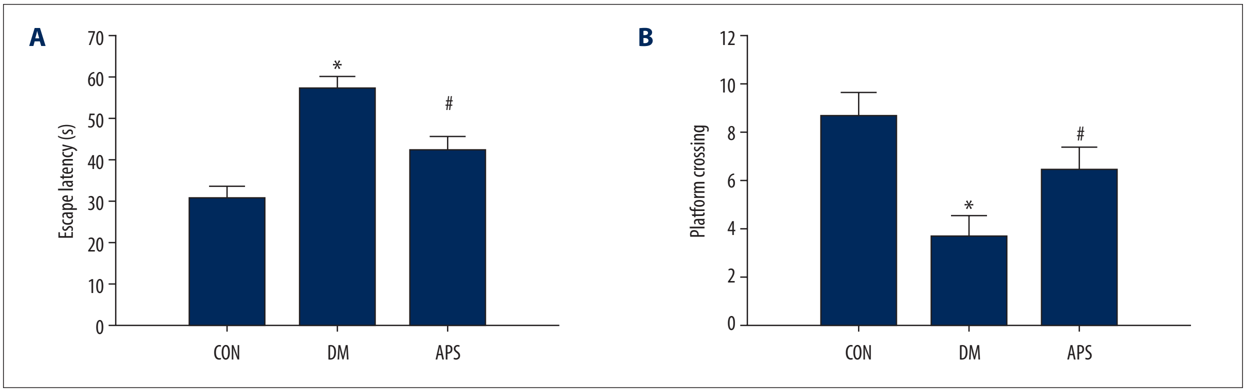

Figure 3. Effect of APS on spatial learning and memory in diabetic rats tested by MWM. (**A**) Changes in the daily escape latencies. (**B**) Time spent in the platform region in the probe trial without the platform. The results are shown as the mean ±SD. Statistical analysis was performed using one-way ANOVA with LSD post hoc test. * p<0.01 *vs.* CON group, # p<0.01 *vs.* DM group.

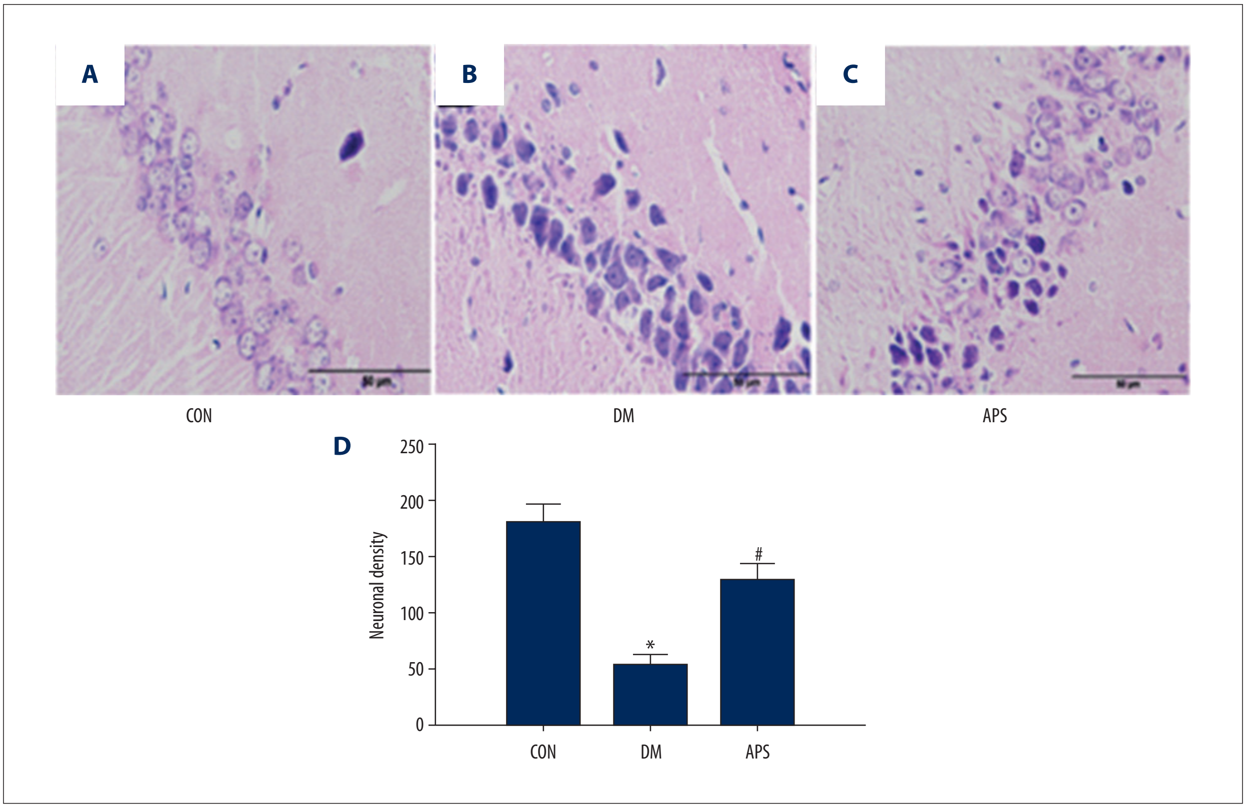

Figure 4. Histological analysis of the effects of APS on neuronal injury induced by diabetes in rats. HE staining was performed on sections of the hippocampal CA1 region. Magnification 40×. (**A**) The neurons in the hippocampal CA1 region of the rats in the CON group were neat and intact, and the cytoplasm and nucleus were full and clearly visible. (**B**) The neurons in the hippocampal CA1 region of the brain were disturbed and loose and the cells became smaller, and there was nuclear pyknosis, chromatin aggregation, and cytoplasm reduction in diabetic GK rat brains. (**C**) APS treatment significantly prevented neuronal cell loss in the hippocampal CA1 region. (**D**) Neuronal density of each group.

Histopathological observations of HE staining showed there were few necrotic cells in the CON group (Figure 4A). In the DM group, the number of neurons in the hippocampus of diabetic rats was decreased, the cell shrinkage was deep, and the nucleoli disappeared (Figure 4B). However, APS administration obviously reversed this alteration (Figure 4C). Neuronal density of each group(Figure 4D).

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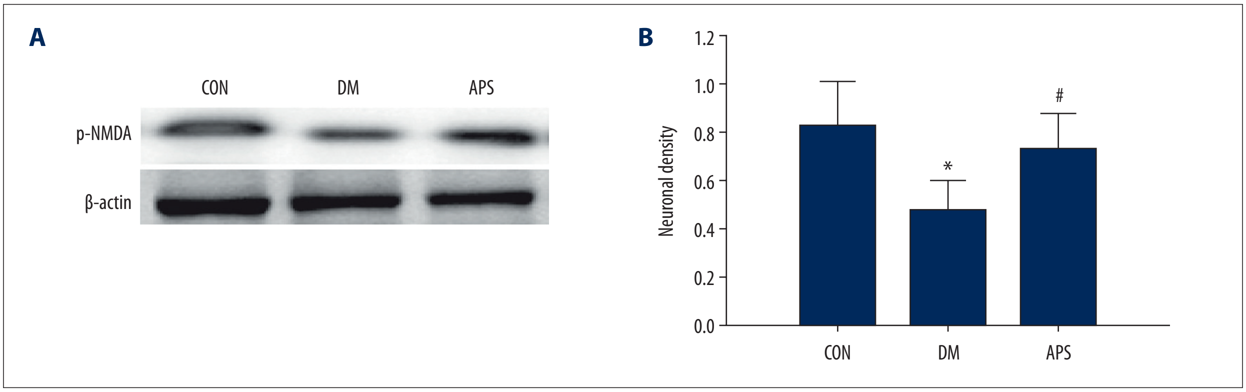

Figure 5. APS increased phosphorylation of p-NMDA receptor. Expression of p-NMDA receptor was detected by Western blot (**A**) and the results are summarized in (**B**). * p<0.01 *vs.* CON group, # p<0.01 *vs.* DM group.

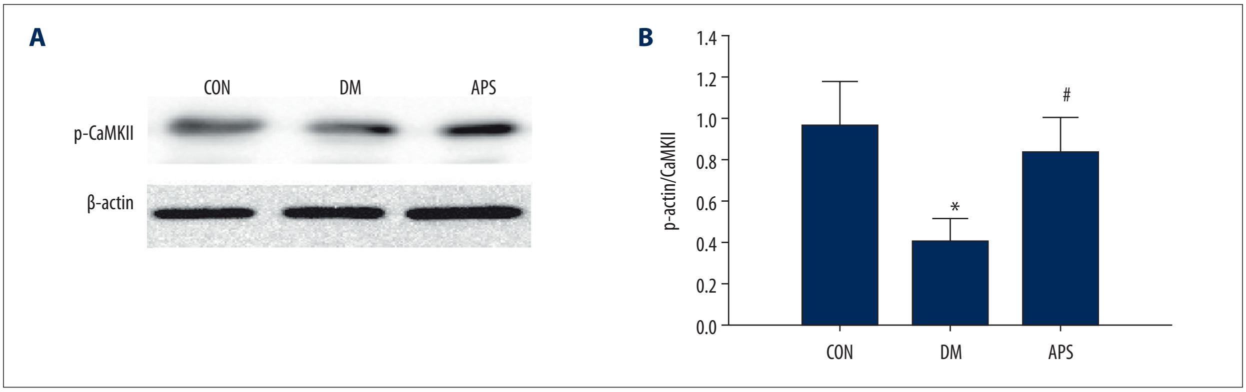

Figure 6. APS increased phosphorylation of p-CaMK II. Expression of p-CaMK II was detected by Western blot (**A**) and the results are summarized in (**B**). * p<0.01 *vs.* CON group, # p<0.01 *vs.* DM group.

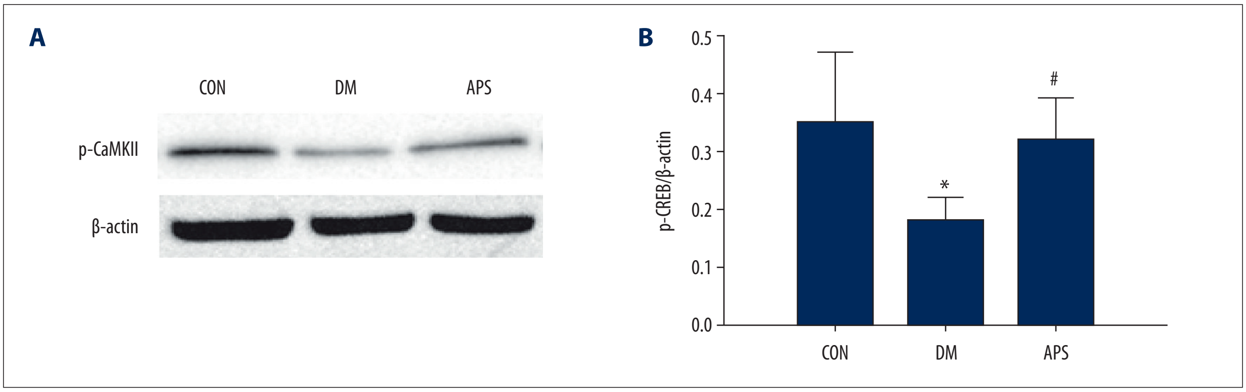

Figure 7. APS increased phosphorylation of p-CREB. Expression of p-CREB was detected by Western blot (**A**), and the results are summarized in (**B**). * p<0.01 *vs.* CON group, # p<0.01 *vs.* DM group.

APS induced changes in expression of p-NMDA receptor, p-CaMK II, and p-CREB. The activity of MDA in diabetic rats was significantly decreased. After APS administration, expression of p-NMDA receptor, p-CaMK II, and p-CREB increased significantly in the hippocampus compared with DM group (Figures 5–7)

Discussion

Diabetes is a major risk factor for cognitive dysfunction, and the risk of developing dementia in diabetic patients is 1.5 times higher than that of non-diabetic patients [17]. Without intervention, the disease will develop into dementia. Dementia is characterized by decreased cognitive function, memory loss, and behavioral changes. Severe cases of diabetes may progress

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to dementia, which imposes heavy financial and psychosocial burdens on the family and community. Therefore, there is urgent need for an effective drug to delay and control the process of diabetic cognitive impairment.

The hippocampus is an important structural functional area of mammalian space learning and memory, and is also a major brain region for neuroplasticity. The vulnerability of hippocampal neurons in the aging process is an important cause of learning and memory degeneration, and the hippocampal nerve function in adults declines rapidly with age.

Synaptic plasticity refers to the changes in morphology and function of synapses, which is the neurobiological basis of learning and memory activities, and plays an important role in development and maturation of the nervous system, as well as learning and memory. Synaptic transmission plasticity is the most critical factor determining synaptic plasticity. Relevant substances that affect outstanding transmission include CREB, NMDA receptor, and CaMKII.

The cAMP response element-binding protein (CREB) is a transcription factor in eukaryotes and plays important roles in the regulation of neurogenesis, synapse formation, learning, and memory. The phosphorylation of CREB is important in regulating transcription, and the transcription of many target genes is activated after the phosphorylation of CREB, which is affected by extracellular signals, showing various physiological functions [18,19].

NMDA receptor is a subtype of ionotropic glutamate receptors. NMDA receptor consists of 7 subunits: NR1, NR2(A, B, C, D), and NR3(A, B). Recent studies have found that there is a close relationship between NMDA receptor subtypes and synapse formation, learning, and memory [20,21].

CaMKII is a major component of postsynaptic compacts (PSD), which can maintain Ca++-independent kinase activity for a longer period of time after autophosphorylation by Ca++ PCaM. The learning and memory ability of CaMKII mutant mice is severely impaired and cannot induce long-term increase (LTP). CaMKII activity increases after learning or LTP induction in mice. Therefore, CaMKII may be the molecular basis of learning and memory [22,23].

APS is a traditional Chinese medicine with a history of thousands of years. It is one of the active ingredients of ginseng, which has various pharmacological and physiological effects. It is well known to people in most parts of the world. APS is the most bioactive constituent in *Astragalus membranaceus* and has been clinically used to treat cancer as an adjunctive medicine strengthening body resistance. APS is also used for treatment of diabetes [24], possibly through regulating the glucose and lipid metabolism and improving insulin resistance. In addition, APS is involved in neuroprotection. Based on the above findings, APS appears to have great potential to reverse memory decline in diabetic animal models.

Our results provide evidence that APS can reduce blood glucose, as well as water and food intake. This is supported by data reported by Changping Dun et al., who showed that APS can treat diabetes through regulating the glucose and lipid metabolism and improving insulin resistance. In addition, the latency to finding the platform in the Morris water maze test was dramatically shortened after APS treatment. Results from the Morris test show that APS can improve learning and memory ability.

CREB, NMDA receptor, and CaMKII are substances that affect outstanding transmission, which is related to synaptic plasticities. In our study, we found that APS could increase the expression of CREB, NMDA receptor, and CaMKII, and cognitive decline was improved in the rat model. APS regulates the expression of CREB, NMDA receptor, and CaMKII which are the synaptic plasticity proteins.

Conclusions

In conclusion, the present study demonstrates that APS improved cognitive decline in a diabetic rat model by regulating the expression of neuroplasticity-associated protein. APS is a traditional Chinese medicine used to treat diabetes and its complications, and its effect is very obvious. In clinical practice it can be used as adjuvant therapy for diabetes.

APS can ameliorate diabetic cognitive decline. The possible mechanism is by regulating the expression of NMDA, CaMK, and CREB.

Conflict of interest

None.

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