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EXPERIMENTAL STUDY

Apocynin ameliorates cognitive deficits in streptozotocin--induced diabetic rats

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ABSTRACT

AIMS: The aim was to investigate the improvement properties of apocynin and its potential mechanism on diabetes-associated cognitive decline.

METHODS: In this study, the model of diabetic rat was established by STZ (50 mg/kg) and treated with apocynin (16 mg/kg/d for 12 weeks). The cognitive ability was evaluated by Morris water maze test. The indicators of oxidative stress (SOD and MDA) were analyzed by spectrophotometer. The inflammatory cytokines were measured by real time-PCR and ELISA. The protein expressions of Nrf-2, HO-1, Bcl-2 and Bax were determined by Western blot.

RESULTS: Treatment with apocynin ameliorated diabetes-related learning and memory injury, as represented by decreasing escape latency and enhancement of the number of times of crossing platform, in the Morris water maze test. In hippocampus, apocynin markedly augmented SOD activity and inhibited MDA level to alleviate oxidative stress. Moreover, apocynin obviously relieved inflammatory reaction by suppressing TNF-α, IL-1β and IL-6 concentrations. Concomitantly, apocynin also statistically enhanced Nrf-2 and HO-1 protein expression to improve DACD. Lastly, apocynin notably ameliorated Bax/Bcl-2 ratio by regulating Bax and Bcl-2 protein expression to mitigate apoptosis.

CONCLUSION: Our results have shown that apocynin may be a valid therapeutic agent against DACD via modulation of antioxidant, anti-inflammatory, and anti-apoptosis *(Tab. 1, Fig. 18, Ref. 35)*. Text in PDF *www.elis.sk*

KEY WORDS: DACD, apocynin, oxidation, inflammation, apoptosis.

Abbreviations: Bax – bcl 2 associated X, Bcl-2 – bcl 2 apoptosis regulator, DACD – diabetes-associated cognitive decline, DM – diabetes mellitus, HO-1 – heme oxygenase, IL-1β – interleukin 1 beta, IL-6 – interleukin 6, MDA – malondialdehyde, Nrf-2 – nuclear factor (erythroid-derived 2)-like 2, SOD – superoxide dismutase, STZ – streptozotocin, TNF-a – tumor necrosis factor

Introduction

Diabetes mellitus is a prevalent metabolism disorder displayed as hyperglycemia. Long-term hyperglycemia is a risk factor of endocrine disease development because of deficiency in insulin resistance and/or release (1). Along with advancement of living standards, the incidence and morbidity of diabetes mellitus increases gradually in humans globally, and the number of diabetic patients is estimated to rise to 592 million in 2035 (2). Diabetes mellitus is associated with many complications such as renal injury, heart disease and brain abnormality. Clinically, the phenotype of brain dysfunction, including cognitive deficits, memory impairments and learning disabilities is universal among diabetic patients (3, 4). Hence, diabetes mellitus has become a great public health issue. The term of diabetes-associated cognitive decline (DACD) has been consistently recognized by health workers and research fellows.

Accumulating evidence implies that cognitive decline involved in diabetes is connected with oxidative stress, inflammatory reaction and apoptosis. It has been proven that oxidative stress plays a vital role in cognitive decline relevant to hyperglycemia (5). Reasonable mechanisms were associated with enhanced level of oxygen free radical and dysfunction of antioxidant defense system. In addition, neuroinflammation was also related to brain diseases in diabetes mellitus (6). TNF-a, IL-1β and IL-6 as inflammatory factors, have been postulated in DACD. More importantly, enhanced oxidative stress and inflammatory reaction might evoke apoptosis in many diseases. Apoptosis signaling is also considered a crucial pathogenic factor in DACD (7). Therefore, modifying oxidative stress, inflammatory reaction and apoptosis pathway is a beneficial strategy for treatment of DACD.

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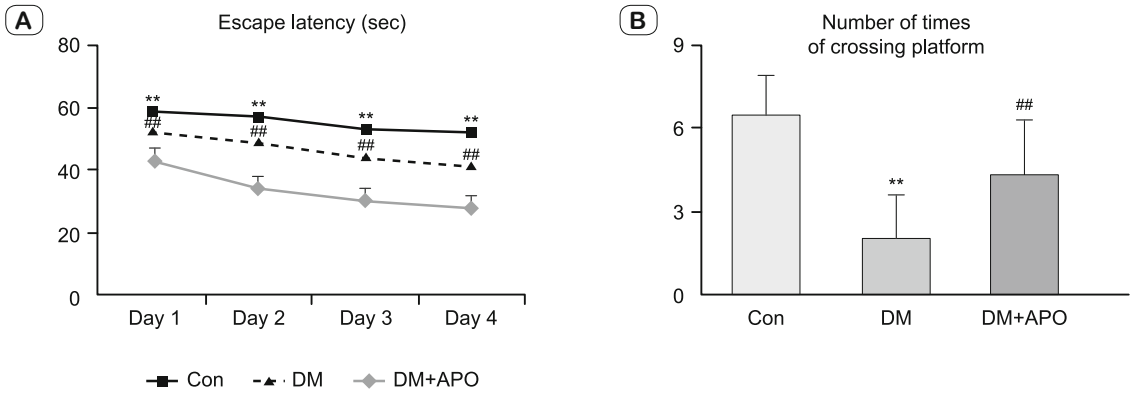

Fig. 1. Properties of apocynin on escape latency (A) and number of times of crossing platform (B) in diabetic rat (n = 15, mean ± SD). ** p < 0.01 compared with Con group; # p < 0.05, ## p < 0.01 compared with DM group. Con, control; DM, diabetic rats; DM +APO, diabetic rats and administered of apocynin (16 mg/kg).

Apocynin, a plant derived drug from Picrorhiza kurroa, is considered as natural medicinal agent for a long time. Recently, anti-oxidation effects of apocynin have been successfully discovered in diabetes mellitus – as represented by enhancing SOD and inhibiting MDA (8). Apocynin is also used as an anti-inflammatory herb which alleviates the expression of inflammasome proteins in brain damage (9). In addition, apocynin restrains apoptotic pathway via regulation of Bax and Bcl-2 expression to improve cognitive impairment and diabetes mellitus (10). However, the precise effect of apocynin in DACD is still elusive. In this study, we investigated the effect and mechanism of apocynin on cognitive impairment induced by streptozotocin in rats.

Materials and methods

Animals

Male Sprague-Dawley rats (about 12 weeks old, 230 ± 20 g) were obtained from the Animal Center of Hunan Normal University (Changsha, China). Rats were maintained at relative constant environment with temperature (23–25 °C), humidity (50–60 %), 12 h light/dark cycle. They were randomly kept in different cages with 3–4 animals. In this study, the experimental protocols were inspected in accordance with the Ethics Committee of Hunan Normal University.

Tab. 1. Primers used in this study (11).

Gene	Accession number		Primer sequences
TNF-α	NM_012675	Forward primer	CCCAGACCCTCACACTCAGAT
		Reverse primer	TTGTCCCTTGAAGAGAACCTG
IL-1β	NM_031512	Forward primer	CACCTCTCAAGCAGAGCACAG
		Reverse primer	GGGTTCCATGGTGAAGTCAAC
IL-6	NM_012589	Forward primer	CCTACCCCAACTTCCAATGCTC
		Reverse primer	TTGGATGGTCTTGGTCCTTAGCC
β-actin	NM_ 031144	Forward primer	CTTCTTGCAGCTCCTCCGTCG
		Reverse primer	TCACACCCTGGTGCCTAGGGC

Drugs and chemicals

Apocynin (Purity ≥ 98.0 %) and streptozotocin (STZ) were obtained from Sangon Biotech (Shanghai, China). The commercial kits of SOD and MDA were purchased from Jiancheng Bioengineering Institute (Nanjing, China). The ELISA kits of TNF-α, IL-1β and IL-6 were supplied by BOSTER Biological Technology (Wuhan, China). The antibodies of Nrf-2, HO-1, Bcl-2, BAX and β-actin were purchased from Proteintech (Wuhan, China). The primer sequences of TNF-α, IL-1β, IL-6 and β-actin were designed by Sangon Biotech (Shanghai, China).

Rat model

Rats were randomly divided into three groups: Control group (CON, n = 15); Diabetes mellitus (DM, n = 15); Diabetes treated with apocynin (DM + APO, n = 15). STZ (streptozotocin, Sigma) was diluted freshly in citrate buffer. Diabetic models were induced via intraperitoneal injection of STZ (50 mg/kg). Only those rats with glycemia ≥ 16.7 mM were regarded as diabetic. Apocynin (Sangon Biotech, Purity ≥ 98.0 %) was orally administered at16 mg/kg/day for 12 weeks.

Morris water maze

Rats were tested by Morris water maze after apocynin treatment to determine the cognitive ability. The period of Morris water maze was 5 consecutive days. At the beginning of the test rats were allowed to swim in a cylindrical tank for 5 min. Each rat swam freely at four starting points to search for hidden platform in 60 sec. The time interval of each experiment was 30 min. The time was measured in each trial to calculate escape latency every day. If rats failed to find the hidden platform, escape latency was regarded as 60 s. At day 5, platform was removed, and rats swam at identical starting placement to search dis-

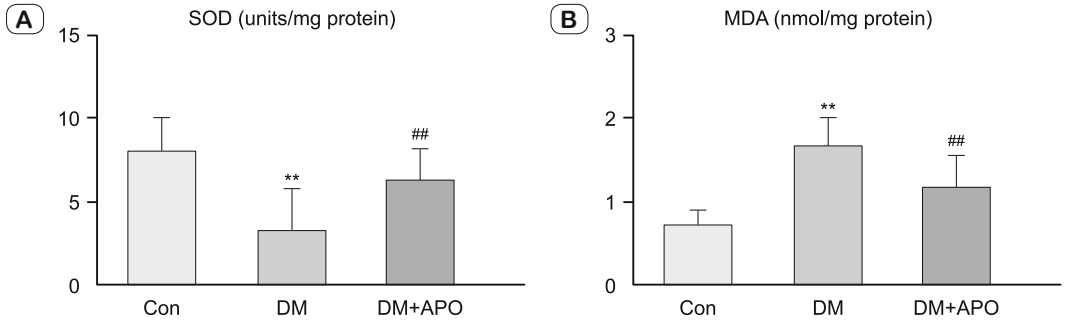

Fig. 2. Effect of apocynin on SOD (A) activity and MDA (B) content in hippocampus of diabetic rats. ** p < 0.01 compared with Con group; ## p < 0.01 compared with DM group. Con, control; DM, diabetic rats; DM + APO, diabetic rats and administration of apocynin (16 mg/kg).

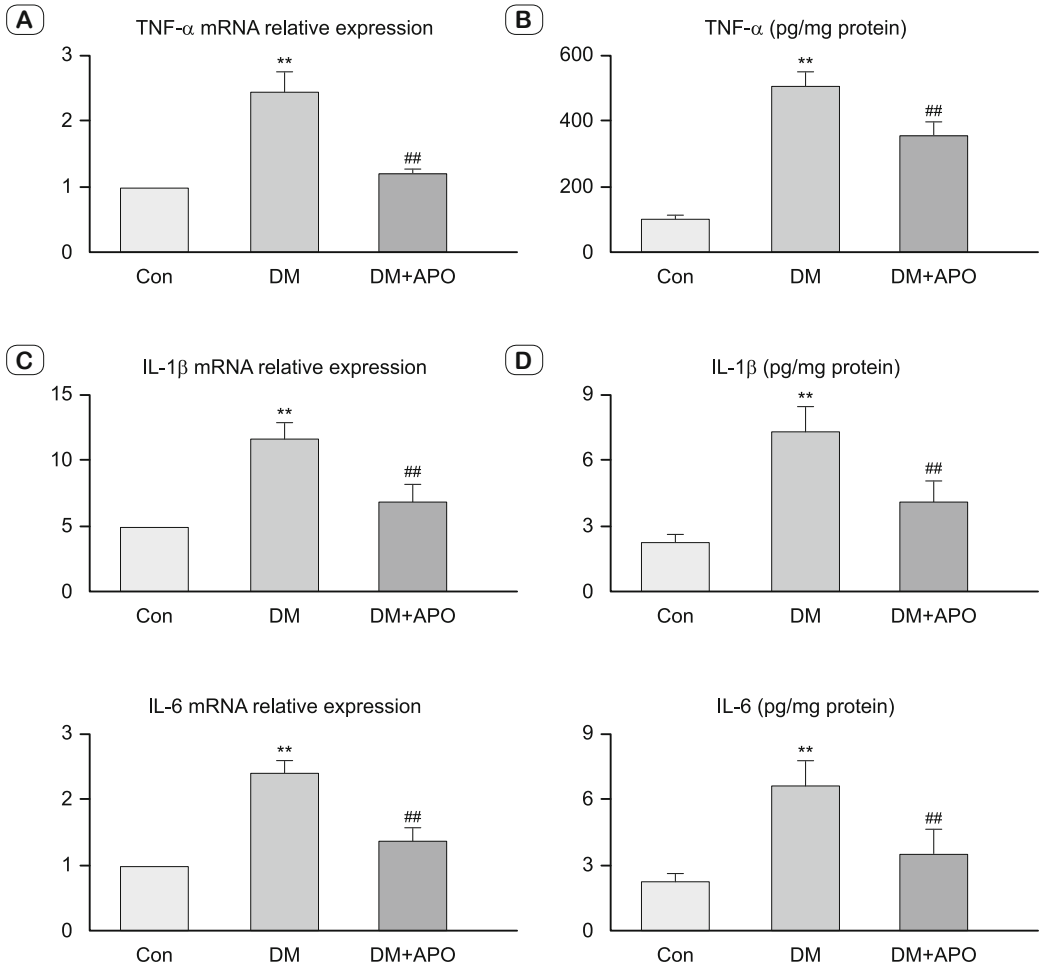

Fig. 3. Effect of apocynin on inflammatory factors including TNF-α (A, B), IL-1β (C, D), and IL-6 (E, F) in hippocampus of diabetes rats. ** p < 0.01 compared with Con group; ## p < 0.01 compared with DM group. Con, control; DM, diabetic rats; DM + APO, diabetic rats and administration of apocynin (16 mg/kg).

appear platform. The frequency of crossing in former placement of platform was recorded.

Biochemical assay

After Morris water maze test, rats were anesthetized with chloral hydrate immediately. Hippocampal sample was surgically dissected from brain and stored at –80 °. Then hippocampus was homogenized, sonicated and centrifuged to extract protein. The biochemical indicators of SOD and MDA were measured by spectrophotometer to evaluate anti-oxidative effect of apocynin. Moreover, ELISA rat kit was used to determine the levels of TNF-α, IL-1β and IL-6 to estimate anti-inflammatory effect of apocynin.

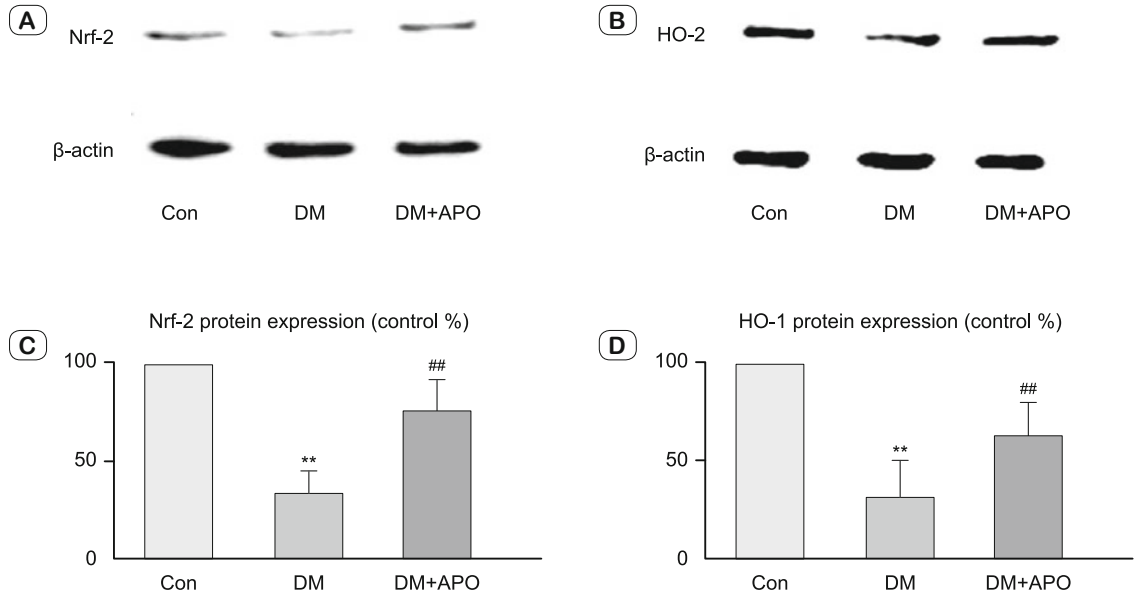

Fig. 4. Effect of apocynin on protein expressions of Nrf-2 (A, B) and HO-1 (C, D) in hippocampus of diabetes rats. ** p < 0.01 compared with Con group; ## p < 0.01 compared with DM group. Con, control; DM, diabetic rats; DM + APO, diabetic rats and administration of apocynin (16 mg/kg).

mRNA gene expression

RNA from Hippocampus was extracted by trizol method and transformed into cDNA. The expression of TNF-α, IL-1β and IL-6 was detected by Bio-Rad CFX real-time PCR. Primers for TNF-α, IL-1β, IL-6 and β-actin are shown in Table 1. The transcriptional expression was calculated via normalizing to β-actin and represented as comparative CT values.

Western blot

Supernatant from hippocampus homogenization was used for appraisement of protein expression. Samples were separated with SDS-PAGE and transferred onto PVDF membranes. Protein was incubated with primary antibody (4°, overnight) and anti-rabbit or anti-mouse secondary antibodies (25°, 2 h). The protein expression was detected by chemiluminescent detection system and visualized with Image J. β-actin was treated as an internal control. A variation was presented relatively by normalizing to β-actin.

Statistics

All results are represented as mean ± SD. The data was analyzed by SPSS 16.0 software. Statistical difference was determined using one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ was deemed statistically significant.

Results

Effect of apocynin on diabetes-induced cognitive deficit

After 12 weeks of apocynin treatment, Morris water maze test was conducted to evaluate learning and memory function in different groups. In comparison to control group, it was remarkable that DM group exhibited longer escape latency, while apocynin noticeably decreased escape latency (Fig. 1A). In the probe trial, the number of times diabetic rats crossed the former platform location was reduced when compared to control group. However, treatment with apocynin obviously enhanced number of times when compared with DM group (Fig. 1B).

Effect of apocynin on oxidative stress in hippocampus of diabetes rats

To explore whether apocynin alleviated oxidative stress, SOD activity and MDA level were detected in hippocampus of diabetic rat. In comparison to control group, the activity of SOD, as one of antioxidant enzymes, was obviously decreased in DM group. Nevertheless, treatment with apocynin markedly augmented SOD activity when compared to DM group (Fig. 2A). In contrast, the content of MDA, as lipo-oxidative product, was distinctly increased in hippocampus of diabetes rats. However, this STZ-induced MDA level was markedly inhibited by apocynin treatment (Fig. 2B).

Effects of apocynin on inflammatory reaction in hippocampus of diabetic rats

To explore the protective function of apocynin on STZ-induced inflammatory reaction, the inflammatory cytokines were detected in hippocampus. As displayed in Figure 3, the contents of TNF-α, IL-1β and IL-6 were obviously enhanced in hippocampus of diabetic rat. However, treatment with apocynin markedly suppressed TNF-α, IL-1β and IL-6 concentrations when compared to DM group.

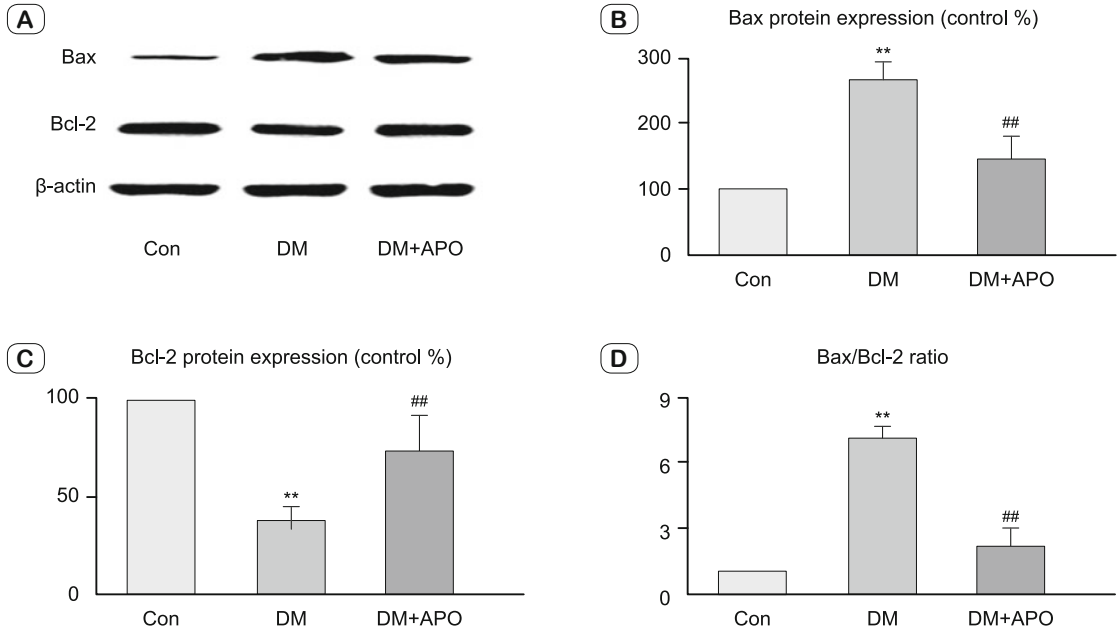

Fig. 5. Effect of apocynin on protein expressions of Bax (A, B), Bcl-2 (A, C) and Bax/Bcl-2 ratio (A, D) in hippocampus of diabetic rats. ** p < 0.01 compared with Con group; ## p < 0.01 compared with DM group. Con, control; DM, diabetic rats; DM + APO, diabetic rats and administration of apocynin (16 mg/kg).

Effect of apocynin on protein expressions of Nrf-2 and HO-1 in hippocampus of diabetic rats

Nrf-2 and HO-1 are well-known regulators closely involved in oxidative stress and inflammatory reactions. To further explore the anti-oxidant and anti-inflammatory roles of apocynin in diabetic rats, Nrf-2 and HO-1 protein expressions were detected in hippocampus. As shown in Figure 4, the protein expressions of Nrf-2 and HO-1 was statistically inhibited in DM group, while treatment with apocynin obviously enhanced Nrf-2 and HO-1 expressions in hippocampus when compared to DM group.

Effect of apocynin on apoptosis response in hippocampus of diabetic rats

To explore anti-apoptotic role of apocynin in diabetes rats, the protein expressions of Bax and Bcl-2 were detected in hippocampus. As shown in Figure 5, it was remarkable that Bax content was markedly increased, while Bcl-2 level was obviously decreased in DM group. In contrast, treatment with apocynin markedly prevented Bax content and augmented Bcl-2 level when compared to DM group. Moreover, apocynin notably regulated Bax/Bcl-2 ratio to improve apoptosis in hippocampus of diabetic rats.

Discussion

Apocynin was widely used as a traditional agent in medicine. In hyperglycemia, apocynin possesses the function of improving diabetic complications, such as renal fibrotic injury, endothelial dysfunction, gastroenteropathy, cardiac damage and neuropathic pain (12–15). In addition, apocynin is involved in neuroprotective properties. Apocynin has been reported to improve learning and memory recovery in many experimental models, such as traumatic brain injury, Alzheimer's disease and Parkinson's disease (16). All these researches demonstrated that apocynin possessed potential efficacy in the therapy of hyperglycemia-related cognitive defects. In this study, apocynin exhibited its beneficial effects in STZ-induced cognitive deficits via ameliorating oxidative stress, attenuating inflammatory response and restraining from apoptosis in the hippocampus.

Oxidative stress is confirmed to be involved in the occurrence of hyperglycemia-induced brain injury (17). Furthermore, oxidative injury is involved in the mechanism of learning and memory deficits. SOD, one of antioxidative enzymes, plays an important role in eliminating ROS in metabolism. MDA, a biomarker of lipid peroxidation, is used as an oxidation index. Previous results showed that apocynin enhanced serum activity of SOD in diabetic rats (18). In learning and memory decline, treatment with apocynin was proved to alleviate MDA level in the model of intermittent hypoxia (19). In this study, apocynin showed its anti-oxidation function to prevent cognitive impairment through enhancing SOD activity and attenuating MDA level in the hippocampus.

Long-term hyperglycemia is a primary mediator of inflammatory reaction and contributes to the development of cognitive deficits. TNF-α, IL-1β and IL-6 were potential contributors to progression of behavioral impairments relevant to diabetes mellitus complications. Previous results showed that puerarin prevented TNF-α and IL-1β to ameliorate streptozotocin-induced cognitive abnormality (20). Moreover, apocynin restored IL-1β and IL-6 expressions to mediating sepsis-induced cognitive dysfunction (21).

In diabetes, apocynin was proved to alleviate retinopathy via inhibiting TNF-a and IL-1β (22). In this study, apocynin treatment could reduce TNF-α, IL-1β and IL-6 activation in the hippocampus, suggesting that apocynin could restrain hyperglycemia-induced cognitive defect via alleviating inflammatory reaction in diabetic rats.

Nrf-2, a crucial transcription element, is a key regulator of antioxidation (23). Nrf-2 can activate antioxidant defense system by regulating antioxidant gene product. Previous results showed that Nrf-2 activation is beneficial for postponing the pathogenic process and ameliorating cognitive deficits in Alzheimer's disease (24). In C57BL/6-Akita diabetic mice apocynin promoted SOD and Nrf-2 expression to suppress oxidative stress (25). In addition, apocynin exhibited its neuroprotective effects in scopolamine-induced brain injury (26). In this study, apocynin increased Nrf-2 expression in hippocampus, which suggested the defense property of apocynin against hyperglycemia-related cognitive function impairment was closely associated with Nrf-2-mediated anti-oxidation effect.

HO-1 is a well-known defensive enzyme and endogenous antioxidant regulator, which also possesses anti-inflammatory effect (27). HO-1 is also a target gene of Nrf-2 and plays a pivotal role in mitigating oxidative stress (28). In long-term diabetes mellitus, the expression of HO-1 was down-regulated (29). Previous results showed that Blueberry anthocyanins protected against retina damage via increasing HO-1 mRNA and protein levels in diabetes (30). In hyperglycemia, apocynin played a key role in retina function via regulating HO-1 level (31). Moreover, apocynin increased expression of anti-inflammatory mediator HO-1 to improve inflammatory bowel disease (32). In this study, apocynin increased HO-1expression in hippocampus possibly via stimulating Nrf-2 pathway, which suggested apocynin protected against neuroinflammation and neurodegeneration in diabetes mellitus.

Bax and Bcl-2 are treated as two crucial indicators of apoptosis dysfunction, which is closely related to process of STZ-induced learning and memory disabilities. Previous results showed that Baicalin modulated pro-and anti-apoptotic regulatory proteins, including Bax and Bcl-2, in hippocampus to relieve STZ-induced cognitive decline (33). In diabetic retinopathy, apocynin increased Bcl-2 expression and decreased Bax expression to attenuate cell apoptosis (22). Furthermore, apocynin reduced cell apoptosis to ameliorate spatial learning impairment in intermittent hypoxia-exposed rats (19). In this study, the protein expression of Bcl-2 was elevated, but Bax was reduced by treatment with apocynin in hippocampus, which suggested apocynin lowered Bax/Bcl-2 ratio to improve apoptosis dysfunction in diabetes-associated cognitive decline.

Conclusion

Apocynin treatment provided beneficial function on learning and memory via appropriate modulation of oxidative response, alleviation of inflammatory reaction and diminution of apoptosis. These results indicate the potential of apocynin as a valid therapeutic agent to relieve cognitive impairment in diabetes mellitus.

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