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*Original Article*

# Apigenin attenuates diabetes-associated cognitive decline in rats via suppressing oxidative stress and nitric oxide synthase pathway

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**Abstract:** Our present investigation aimed to determine the neuroprotection of apigenin (API) against diabetes-associated cognitive decline (DACD) a diabetic rat model and exploring its potential mechanism. Diabetic rat model was induced by intraperitoneal injection of streptozotocin. All experiment animals treated with vehicle or API by doses of 10, 20 and 40 mg/kg for seven weeks. Firstly, the body weight and blood glucose levels were detected. We used Morris water maze test to evaluate learning and memory function. The oxidative indicators (malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH)), cNOS, iNOS, caspase-3 and caspase-9 were measured in cerebral cortex and hippocampus using corresponding commercial kits. API can increase body weight, reduce the blood glucose levels, and improve the cognitive function in rats induced by diabetes. API decrease the MDA content, and increase SOD activity and GSH level of diabetic animals in the cerebral cortex and hippocampus of diabetic rats. Meanwhile, constitutive nitric oxide synthase (cNOS), inducible nitric oxide synthase (iNOS), caspase-3/9 were markedly exhibited in the cerebral cortex and hippocampus of diabetic rats. In summary, our current work discloses that API attenuates DACD in rats via suppressing oxidative stress, nitric oxide and apoptotic cascades synthase pathway.

**Keywords:** Apigenin, diabetes, diabetes-associated cognitive decline, oxidative stress, nitric oxide

## Introduction

Diabetes is a systemic disease that can cause a range of complications, which can affect the structure and function of the central nervous system through different stages, and their effects may overlap and magnify [1]. Diabetes-associated cognitive decline (DACD) is not caused by only one factor, but the multi-factor and multi-link complex pathological changes [2].

Oxidative stress degree is in proportion to the number of neuronal mitochondrial DNAs [3, 4]. It is found that mitochondrial enzymes of brain cells in DACD mouse are damaged. Due to the mitochondrial dysfunction, more radicals may be released, and with the oxidizing effect of lipids on the cell membrane and the formation of aldehydes toxic substances, oxidative stress occurs more frequently in neuron of DACD patients [5].

In recent years, the role of NO in the effects of diabetes on the central nervous system is attracting increasing attention, which is considered to be a connection between diabetes neuropathy vascular doctrine and metabolic doctrine [6]. Excessive or inadequate generation of NO is the cause or contributing factor to many diseases [7]. The regulations of the body on the different functions of NO are mainly realized by the fine regulation of different nitric oxide synthase (NOS) [8, 9]. Studies have proved that NO is involved in the development processes of diabetes and a variety of chronic complications. But the changes of NO and NOS in brain tissue during diabetes, as well as their role in the pathogenesis of diabetes in the central nervous system lesions are not clear [10].

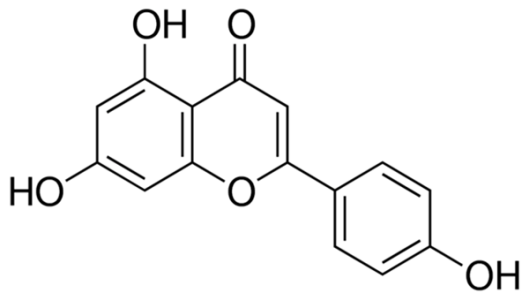


**Figure 1.** The chemical structure of API.

Apigenin (API), as a naturally existing flavonoid, has been found that it has a variety of pharmacological activities, mainly including anti-tumor, anti-oxidative stress, anti-DNA damage and so on [11]. API can inhibit the protective effect of oxidative stress on the melanin apoptosis induced by $H_2O_2$ by reducing reactive oxygen species (ROS) generation [12]. API can decrease the expressions of iNOS mRNA and protein, thereby inhibiting the synthesis of NO [13]. In our present study, we aimed at examining whether API has a protective role against DACD in rats via suppressing oxidative stress and nitric oxide synthase pathway. Therefore, the present investigation was designed to evaluate the neuroprotection of API on DACD using a diabetic rat model and further figure out the potential mechanisms.

## Materials and methods

### *Drugs and chemicals*

API (with a purity > 97%) and Streptozotocin (STZ) were purchased from Sigma (St. Louis, MO, USA). The chemical structure of API was displayed in **Figure 1**. Malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH), constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS) ELISA kits were provided Jiancheng Biotech Co (Nanjing, China). All other chemical reagents were of analytical grade.

### *Animals and ethics statement*

Male Wistar rats (240 ± 10 g) were acquired from Laboratory Animal Care of Universities of Shandong. All experiment animals were fed food and water ad libitum in a room where lighting was controlled on a 12-h light/dark cycle and 24 ± 2°C with 65 ± 5% humidity. Great efforts were made to minimize the number of the animals used and the suffering.

### *Induction and measurement of diabetes*

After 1 week acclimatization, diabetes was induced by a single intraperitoneal injection of STZ (Sigma, St Louis, USA) dissolved in 0.1 M sodium citrate buffer (pH 4.5), at a dose of 60 mg/kg. The STZ injection day was appointed as day zero and 72 h later, fasting blood glucose levels were monitored and all rats with fasting blood glucose levels > 250 mg/dL were considered diabetic and used for further.

### *Group design*

All experiment animals were randomly divided into 5 groups: (1) control group (Con) (n = 8), which was the normal rats and received physiological saline (0.1 ml/100 g) intraperitoneally (i.p.); (2) vehicle group (DM) (n = 8), which was the diabetic rats and received physiological saline (0.1 ml/100 g) i.p.; (3-5) API groups (DM + API (10), DM + API (20) and DM + API (40)) (n = 8), which were diabetic rats and treated with API at doses of 10, 20 and 40 mg/kg i.p., respectively. API and physiological saline were dissolved once a day. The control group and vehicle group received vehicle of API from the third day of experiment until seventh week.

### *Morris water maze*

After seven weeks, Morris water maze for five consecutive days was used to evaluate the learning and memory functions of all groups. The chloral hydrate (300 mg/kg) was injected into the experiment animals for anesthesia. Under anesthesia, the rats for each group were sacrificed, then the brains were rapidly removed and blood samples were collected. The samples were stored at -80°C until used for further experimentation measurements.

The apparatus consisted of a circular water tank (50-cm in height × 90-cm diameter), filled with water (26 ± 1°C) made opaque with milk power. On the first day, all rats were given a 120 s habituation session to the water by being allowed to swim freely in the pool without the platform, prior to performing the water maze test. Over the following 4 days, each rat underwent four 120 s learning trials with the platform at intervals of 60 s. Then, rats were placed into

**Table 1.** The body weight and blood glucose levels in the four groups of rats at the onset and at the end of the experiment

| Treatment | Body weight (g) | | Plasma glucose (mg/dl) | |
|---|---|---|---|---|
| | Onset of study | End of study | Onset of study | End of study |
| Con | 242.20 ± 3.89 | 295.60 ± 4.92 | 115.10 ± 1.23 | 110.10 ± 1.21 |
| DM | 245.90 ± 7.31 | 143.6 ± 3.72** | 111.80 ± 1.41 | 591.20 ± 3.79** |
| DM + API (10) | 237.80 ± 5.14 | 232.70 ± 5.11## | 106.80 ± 1.82 | 305.20 ± 3.62## |
| DM + API (20) | 242.30 ± 5.11 | 253.20 ± 5.42## | 107.20 ± 2.51 | 298.30 ± 3.45## |
| DM + API (40) | 244.20 ± 5.16 | 267.20 ± 5.87## | 108.50 ± 2.61 | 291.50 ± 3.31## |

**P < 0.01 compared with Con group; ##P < 0.01 compared with DM group. Con, control group; DM, diabetes group; DM + API (10), apigenin (10 mg/kg)-treated group; DM + API (20), apigenin (20 mg/kg)-treated group; DM + API (40), apigenin (40 mg/kg)-treated group.

the water facing the pool wall at one of four points of entry, namely, labeled N (north), S (south), E (east) and W (west). The escape latency was recorded for each trial. If a rat was unable to locate the platform within 120 s, it was led to the platform and allowed to rest there for 60 s. The escape latency in these cases was recorded as 120 s. A translucent acrylic platform was submerged 1 cm below the water surface and removed from the tank. The water maze task was performed daily for 6 days. After the final escape training, the platform was removed and each rat was placed into the pool from the start location at the quadrant opposite to the former platform quadrant. The number of times of crossing the former location of the platform and the time spent in the former platform quadrant were analyzed with an interval of 60 s.

*Measurement of oxidative stress*

The malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) concentrations were measured in homogenized the samples of cerebral cortex and hippocampus with fluorescence detection. In according to the manufacturer's instructions (Jiancheng Biotech Co., Nanjing, China), the MDA contents, SOD activity and GSH level were monitored at 532, 450nm and 340 nm by MDA, SOD and GSH ELISA kits, respectively.

*Measurement of activities of cNOS and iNOS*

Samples of cerebral cortex and hippocampus were fixed in 10% buffered formalin. In accordance with manufacturer's instructions (Jian-Cheng Bioengineering Institute, Nanjing, China), expressions of cNOS and iNOS were detected immunohistochemically by cNOS and iNOS assay kit. The photometric measurement of the absorbance at 530 nm was applied to determine cNOS and iNOS activity.

*Measurement of the activities of caspase-3 and caspase-9*

Caspase-3 and caspase-9 are regarded as an executioner molecule in the apoptotic cascades. The activity of caspase-3 and caspase-9 were analyzed by cleavage of chromogenic caspase substrates, Ac-DEVD-pNA. In accordance with instructions of manufacturer (R&D Systems, USA), the amount of and caspase-9 were measured at 405 nm by spectrophotometer.

*Statistical analysis*

Data were presented as means ± S.D. For statistical comparisons, the results were analyzed using one-way ANOVA followed by Dunnett's test. A $p$-value < 0.05 was considered the significant level.

## Results

*Effect of API on body weight and blood glucose levels*

After 21-day treatment, the value of the body weight in the DM group was markedly reduced form 245.90 ± 7.31 to 143.6 ± 3.72 g, compared to the control group (P < 0.01), as shown in **Table 1**. However, after 21-day treatment with API by the doses of 10, 20 and 40 mg/ml, the value of the body weight was significantly reversed in diabetic rats from 237.80 ± 5.14 to 232.70 ± 5.11 g (P < 0.01), 242.30 ± 5.11 to 253.20 ± 5.42 g (P < 0.01) and 244.20 ± 5.16 to 267.20 ± 5.87 g (P < 0.01), respectively, compared to the DM group (P < 0.01), as shown

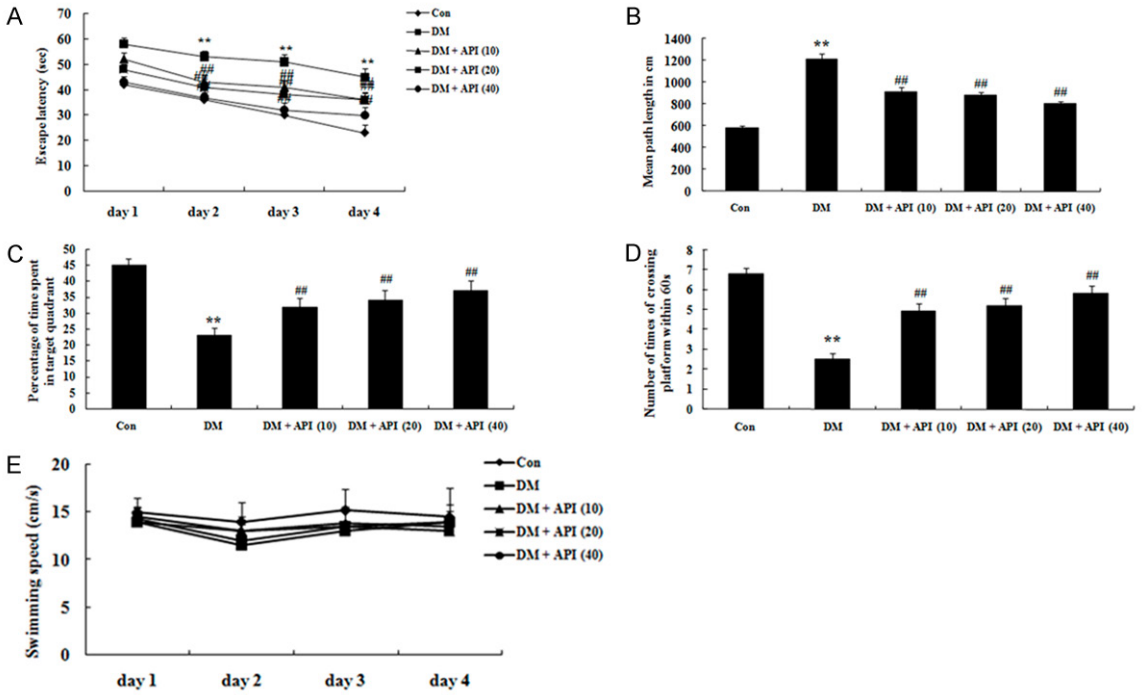


**Figure 2.** Effect of API on diabetes-induced cognitive deficit. Effect of API on the escape latency (A) mean path length (B) mean percentage of time spent in the target quadrant (C) the number of times of crossing platform (D) and swimming speed (E). $^{**}P < 0.01$ compared with Con group; $^{\#\#}P < 0.01$ compared with DM group. Con, control group; DM, diabetes group; DM + API (10), API (10 mg/kg)-treated group; DM + API (20), API (20 mg/kg)-treated group; DM + API (40), API (40 mg/kg)-treated group.

in **Table 1**. In the meantime, the levels of blood glucose in the DM group was significantly increased from 111.80 ± 1.41 to 591.20 ± 3.79 mg/dl, compared to the control group ($P < 0.01$), as shown in **Table 1**. After 21-day treatment with API by the doses of 10, 20 and 40 mg/ml, the levels of blood glucose was significantly prevented in diabetic rats from 106.80 ± 1.82 to 305.20 ± 3.62 g ($P < 0.01$), 107.20 ± 2.51 to 298.30 ± 3.45 g ($P < 0.01$), and 108.50 ± 2.61 to 291.50 ± 3.31 g ($P < 0.01$), respectively, compared to the DM group ($P < 0.01$), as shown in **Table 1**.

*Effect of API on diabetes-induced cognitive deficit*

The Morris water maze test was used to assess the cognitive function (21th day). There was a significant reduction of the escape latency in the DM rats from second day to 4th day training, when compared to that of control rats (**Figure 2A**). When compared to DM rats, treatment with API by the doses of 10, 20 and 40 mg/ml could exhibit shorter escape latency from second day to 4th day training (**Figure 2A**). In the probe test, the mean path length of DM rats was significantly longer than that of control rats at 5th day training (**Figure 2B**). The results revealed a significant reduce of DM/API rats (10, 20 and 40 mg/ml) at 5th day training, when compared to that of DM rats (**Figure 2B**).

In addition, analysis of the time spent in the target quadrant revealed that there was an evident decline of the time spent in the target quadrant in DM rats, when compared to that of control rats (**Figure 2C**). When compared with DM rats, the time spent in the target quadrant of DM/API rats (10, 20 and 40 mg/ml) during the 4 days of training was significantly higher in DM rats (**Figure 2C**). In the meantime, we also observed that the number of times the animals crossed the former platform location of DM rats was also lower as compared to those in control group (**Figure 2D**). After administration of API treatment by the doses of 10, 20 and 40 mg/ml spent more time, API treatment could significantly mitigate these changes (**Figure 2D**). Nevertheless, there was no significant difference in swimming speed in all experimental groups (**Figure 2E**).

**Table 2.** The MDA, SOD and GSH levels in the cerebral cortex and hippocampus of rats from each group

| Treatment | MDA (nmol/mg protein) | | SOD (units/mg protein) | | GSH (mol) | |
|---|---|---|---|---|---|---|
| | Cerebral cortex | Hippocampus | Cerebral cortex | Hippocampus | Cerebral cortex | Hippocampus |
| Con | 1.22 ± 0.11 | 0.90 ± 0.08 | 8.87 ± 0.06 | 8.71 ± 0.09 | 33.00 ± 0.39 | 31.89 ± 0.63 |
| DM | 2.61 ± 0.07** | 1.61 ± 0.07** | 4.11 ± 0.12** | 3.91 ± 0.10** | 16.08 ± 0.47** | 14.72 ± 0.23** |
| DM + API (10) | 2.12 ± 0.05## | 1.36 ± 0.05## | 7.88 ± 0.06## | 7.66 ± 0.06## | 28.16 ± 0.81## | 26.81 ± 0.70## |
| DM + API (20) | 1.98 ± 0.04## | 1.30 ± 0.05## | 7.97 ± 0.06## | 7.87 ± 0.04## | 29.31 ± 0.73## | 27.94 ± 0.73## |
| DM + API (40) | 1.85 ± 0.05## | 1.22 ± 0.06## | 8.18 ± 0.08## | 8.02 ± 0.06## | 30.48 ± 0.89## | 29.31 ± 0.88## |

**P < 0.01 compared with Con group; ##P < 0.01 compared with DM group. Con, control group; DM, diabetes group; DM + API (10), apigenin (10 mg/kg)-treated group; DM + API (20), apigenin (20 mg/kg)-treated group; DM + API (40), apigenin (40 mg/kg)-treated group.

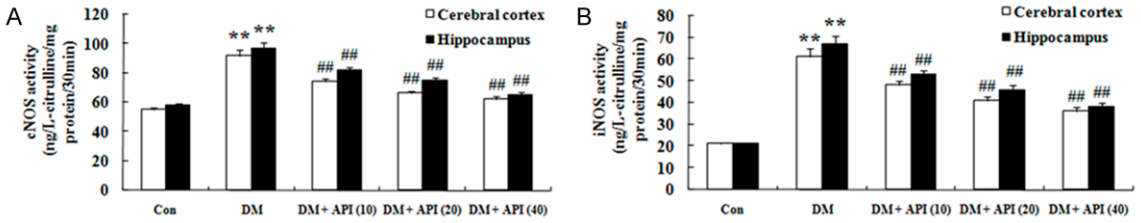


**Figure 3.** Effect of API on the activities of cNOS and iNOS. Effect of API on diabetes-induced changes in the activities of cNOS and iNOS (A) and (B). **P < 0.01 compared with Con group; ##P < 0.01 compared with DM group. Con, control group; DM, diabetes group; DM + API (10), API (10 mg/kg)-treated group; DM + API (20), API (20 mg/kg)-treated group; DM + API (40), API (40 mg/kg)-treated group.

*Effect of API on diabetes-induced changes in oxidative stress*

After 21-day treatment, **Table 2** illustrated that the MDA contents of the DM group was significantly increased both in cerebral cortex and hippocampus, in comparison to the control group (P < 0.01). Simultaneously, in cerebral cortex and hippocampus, chronic administration of API (10, 20 and 40 mg/ml) remarkably reversed the elevation of MDA content, in comparison to the DM group (P < 0.01). However, SOD activity and GSH level of diabetic animals were both found to be evidently decreased in cerebral cortex and hippocampus, in comparison to the control group (P < 0.01). After 21-day treatment of API at the doses of 10, 20 and 40 mg/ml, this reduction of SOD activity and GSH level were significantly prevented in cerebral cortex and hippocampus of the DM/API group (P < 0.01), as shown in **Table 2**.

*Effect of API on diabetes-induced changes in the activities of cNOS and iNOS*

**Figure 3A**, **3B** illustrated that the activities of cNOS and iNOS in the DM group were significantly elevated in cerebral cortex and hippocampus of diabetic rats after 21-day treatment (P < 0.01). However, supplement with API (10, 20 and 40 mg/ml) remarkably and dose dependently inhibited the activities of cNOS and iNOS in cerebral cortex and hippocampus of the DM/API group (P < 0.01).

*Effect of API on the activities of caspase-3 and caspase-9*

**Figure 4A**, **4B** illustrated that the activities of caspase-3 and caspase-9 in the DM group were significantly elevated in cerebral cortex and hippocampus of diabetic rats after 21-day treatment (P < 0.01). However, supplement with API (10, 20 and 40 mg/ml) remarkably inhibited the activities of caspase-3 and caspase-9 in cerebral cortex and hippocampus of the DM/API group (P < 0.01).

## Discussion

With the increasing proportion of older people, and the prolonged average life expectancy, the numbers of DACD and dementia patients are also increasing [14]. Impact of diabetes on the

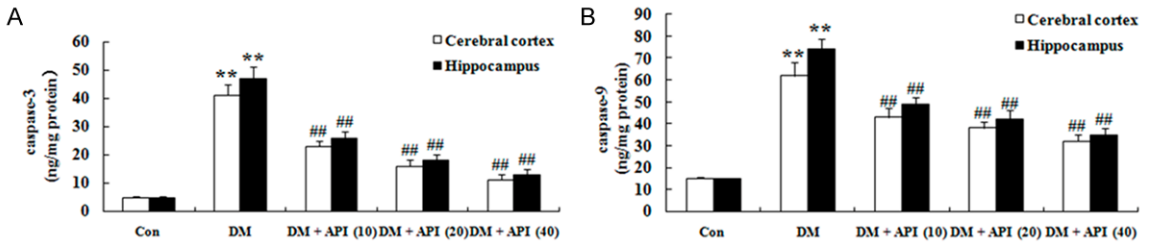


**Figure 4.** Effect of API on the activities of caspase-3 and caspase-9. Effect of API on the activities of caspase-3 caspase-9 (A) and (B). $^{**}P < 0.01$ compared with Con group; $^{\#\#}P < 0.01$ compared with DM group. Con, control; DM, diabetes; DM + API (10), API (10 mg/kg)-treated group; DM + API (20), API (20 mg/kg)-treated group; DM + API (40), API (40 mg/kg)-treated group.

central nervous system includes increasing the incidence of cerebrovascular disease and multiple aggravated harm, and leading to chronic diabetic encephalopathy with DACD as prominent feature [15, 16]. Our present study firstly demonstrated that API can increase body weight, reduce the blood glucose levels, and improve the cognitive function in rats induced by diabetes. A recent research illustrated that API could exert protection against kainate-induced excitotoxicity by anti-oxidative effects, which was partly agreement with our findings [17].

In the brain microvasculature and brain issues of rats with API, glucose level is increased, and lipid peroxide content is increased, resulting in oxidative damage. In addition, high blood sugar activates protein kinase C (PKC), producing oxidative stress that can further enhance PKC activity through the activation of poly-ADP-ribose polymerase (PARP), thus forming a vicious cycle of oxidative stress, and causing brain oxidative stress injury of diabetes. ROS can be quickly scavenged by endogenous antioxidant enzymes such as SOD and low-molecular weight antioxidants, such as GSH [18]. However, an excessive production of ROS during diabetic conditions contributes to the relative reduced capability of the natural antioxidant systems leading to the neuronal apoptosis. Currently there are many synthetic antioxidants, while API abounds in our daily ingestion of fruits, vegetables, legumes and tea, which is one of the most antioxidant foods with free-radical elimination and DNA-damage prevention characteristics [19]. The experimental results also show that, API can significantly weaken the increase of the melanin cells ROS content induced by $H_2O_2$. It is suggested that the anti-apoptotic effect of API may be related to the antioxidant effect [20]. Our current investigation illustrated that the MDA content was decreased, and SOD activity and GSH level of diabetic animals were all increased in the cerebral cortex and hippocampus of diabetic rats, which was consistent with the previous report [21, 22].

In recent years, the role of NO in the central nervous system has received increasing attention, considered to be is considered to be a connection between Diabetes neuropathy vascular doctrine and metabolic doctrine. NO in vivo is generated by being catalyzed by NOS, cNOS and iNOS are two isoforms of NOS, rarely expressed under normal circumstances [23, 24]. Diabetes can induce the increased production of cNOS and iNOS, and then lead to increased production of NO; excessive NO possesses neurotoxic effect. API can significantly increase total NOS activity in ischemia-reperfusion myocardial tissue, suggesting that API elevates ischemia reperfusion plasma and NO content of myocardial tissue by increasing the total NOS activity of the myocardial tissue [10, 14]. Because in this study, only finding API has no significant effect on iNOS activity of myocardial tissue, suggesting that API improves eNOS activity myocardial tissue by protecting endothelial cells [25]. Our present study indicated that API significantly and dose-dependently treated DACD via suppressing oxidative stress and NO signaling. In consistent with our results, API can inhibit α-glucosidase activity and supply moderate NO for preventing the development of diabetic complications.

Caspases are specifically activated with the apoptotic stimuli and caspase-3/9 is conceived of as an executioner in apoptotic cascades. In

the present study, API treatment markedly exhibited the elevation of caspase-3/9 activity in cerebral cortex and hippocampus n a rat model of diabetes. And this effect of API reduced neuronal cell death in a rat model of diabetes. In the meantime, it was previously found that API can induct the apoptosis and relate flavonoids through activation of caspase-9 and caspase-3 in leukaemia HL-60 cells [26].

In summary, our current work depicted that API treatment could provide beneficial effects by means of decreasing blood glucose, improving learning and memory functions, inhibiting oxidative stress, reducing the NO and diminishing caspase-3/9 activity in diabetes rat. These data point indicates that API attenuates DACD in rats via suppressing oxidative stress and nitric oxide synthase pathway.

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## Disclosure of conflict of interest

None.

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