

## Original Article

# Baicalein alters PI3K/Akt/GSK3 $\beta$ signaling pathway in rats with diabetes-associated cognitive deficits

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**Abstract:** Our present investigation focused on assessing the neuroprotective potential of baicalein (BAC) against diabetes-associated cognitive deficit (DACD) using a diabetic model and further figure out the potential molecular mechanisms. Diabetic rat model was established by streptozotocin (STZ). Vehicle or BAC by the doses of 2 and 4 mg/kg was intraperitoneally injected once a day for seven consecutive weeks. Memory function was evaluated by Morris water maze test and avoidance passive test. The activities of acetylcholinesterase (AChE), choline acetylase (ChAT), caspase-9 and caspase-3 in STZ-induced diabetic rats' hippocampus were detected via responsive commercial kits. Western blot assay were used to determine the protein levels of phospho-phosphatidylinositol 3-kinase (p-PI3K), phospho-Akt (p-Akt), and phospho-glycogen synthase kinase-3 $\beta$  (p-GSK3 $\beta$ ). Our results showed that BAC remarkably increased body weight and ChAT activity, decreased blood glucose level and AChE activity as well as improved cognitive deficits in diabetic rats. Additionally, it was also found that treatment with BAC to diabetes obviously stimulated the p-PI3K and p-Akt and inhibited the level of p-GSK3 $\beta$ . Furthermore, the neuronal apoptosis was also prevented after BAC treatment by decreasing caspase-9 and caspase-3 activities in diabetic rats' hippocampus. It is concluded that BAC exerted beneficial effects against DACD in rats and its neuroprotection might be linked with activating PI3K and Akt phosphorylation accompanied with suppressing the phosphorylated level of GSK3 $\beta$ . These results hint that BAC is likely to be served as an adjuvant therapy to conventional anti-hyperglycemic regimens as well as DACD.

**Keywords:** Baicalein, diabetes-associated cognitive decline, neuroprotection, phosphatidylinositol 3-kinase, Akt, glycogen synthase kinase-3 $\beta$

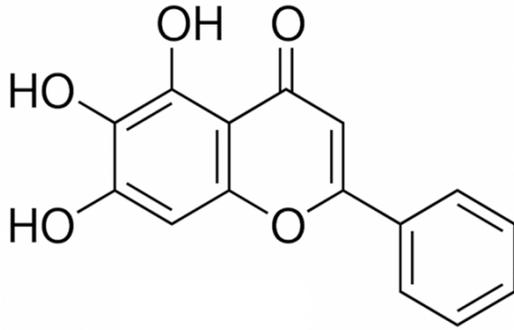
## Introduction

Ample evidence illustrates that diabetes mellitus (DM) can adversely influence central nervous system (CNS) and cognitive deficit is conceived of as the most common symptom [1]. Indeed, it was estimated that diabetic patients had a double risk for the occurrence of dementia in 6370 elderly individuals [2]. In preliminary investigations, the rats with diabetes-associated cognitive decline (DACD) exhibited marked neuronal loss and exacerbated brain damage [3-5]. These findings implicate that DACD is a very serious problem and it is of desperate need to figure out the underlying molecular mechanism and develop the novel therapeutic target for the prevention of these cognitive symptoms.

Although cognitive dysfunction in diabetes may be caused by a variety of factors [6], phospho-

phatidylinositol 3-kinase (PI3K)/Akt/glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) is regarded as the most important signaling pathway. The PI3K/AKT/GSK3 $\beta$  pathway is crucial to maintenance of the neuronal network and cell survival. Concurrently, it was previously found that the activation of PI3K/Akt significantly reduced the neuronal damage in ischemic stroke [7]. Additionally, the activation of GSK3 $\beta$  was also observed in vitro cognition-deficient cell model caused by A $\beta$  peptides [8]. Suppression of GSK3 $\beta$  could remarkably diminish A $\beta$  deposition and finally alleviate spatial learning and memory deficits in transgenic mice with Alzheimer's disease [9]. It suggests that modulation of PI3K/Akt/GSK3 $\beta$  may be served as a very important target for the amelioration of DACD.

Baicalein (BAC) is an important medicinal herb purified from the root of *Scutellaria baicalensis*



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

*Georgi* and has been reported to exhibit anti-oxidative [10] and anti-inflammatory [11] properties. A previous study demonstrated that BAC had a protective role against ischemia-reperfusion injury in a chick embryonic ventricular myocyte model [12]. What's more important, it was found that BAC could improve cognitive deficits induced by chronic cerebral hypoperfusion in rats [13]. However, whether BAC exerts protection against DACD remains elusive. Besides, as PI3K/Akt/ GSK3 $\beta$  plays a crucial role in the generation of learning and memory function, we hypothesize that it involves in the BAC's neuroprotection. The present work focused on evaluating the protective effect of BAC against DACD using a streptozotocin (STZ)-diabetic rat model and further figure out the potential molecular mechanisms.

## Materials and methods

### Animals

Adult male Wistar rats (230-250 g) were obtained from the breeding colony by the animal center of Chinese Academy of Sciences in China) and maintained under a controlled environment at a constant temperature (24°C), humidity (50-70%) and light/dark (12:12 h) cycle. They were allowed to give free access to standard diet and water. Throughout our study, the experimental protocols were approved by Ministry of Health PR China and Animal Care Committee of the First Affiliated Hospital of Dalian Medical University.

### Preparation of experimental diabetes and drug treatment

The experimental DM was established by intraperitoneal injection of a single dose of 65 mg/kg STZ dissolved in citrate buffer (pH 4.4, 0.1

M). The control rats an equal volume of citrate buffer only. Blood samples were collected 48 h after STZ injection. Rats with fasting plasma glucose levels higher than 250 mg/dl [14] were considered as diabetic and selected for further investigation. The rats were randomly divided to four groups as follows (n = 10 for each group): non-diabetic control (Con), diabetic rats (DM), diabetic rats treated with 2 mg/kg BAC per day (BAC (2)) and diabetic rats treated with 4 mg/kg BAC per day (BAC (4)). The dosage and dosing frequency of BAC were chosen as previously described [15, 16]. BAC (with a purity > 98%, Sigma, St. Louis, MO, USA) was freshly prepared by dissolving in the DMSO and injected intraperitoneally once a day for seven weeks after diabetes induction.

### Morris water maze test

Seven weeks later, Morris water maze tests were performed to evaluate the learning and memory functions of rats from different groups. The apparatus consisted of a circular water tank (180 cm in diameter and 60 cm in height) filled with water (24°C). A translucent platform, invisible to the rats, was submerged approximately 1 cm below the water surface (for the navigation test) or removed from the tank (for the spatial probe test). The water maze test was carried out for 5 consecutive days. Learning test was evaluated according to the previous method [17]. For short, animals were subjected to 4 consecutive daily training trials for 4 days. For each trial, the rats had to swim until it climbed onto the platform. The animals then stayed on the platform for 20 s before the commencement of the next trial. The escape latency (s) and path length (cm) to find the platform were calculated in each trial and averaged over three trials for each animal. Concurrently, swimming speed was calculated by dividing the path length by the time to find the platform. 24 h after the last learning session, 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 recorded with an interval of 60 s during the probe trials.

### Passive avoidance test

Animals also experienced the passive avoidance test according to the previous descrip-

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**Table 1.** Effect of BAC on body weight and blood glucose levels (n = 10, mean  $\pm$  S.D.) 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	242.11 $\pm$ 3.54	295.67 $\pm$ 5.07	111.24 $\pm$ 2.57	108.63 $\pm$ 1.54
DM	244.38 $\pm$ 5.91	132.31 $\pm$ 2.89**	113.45 $\pm$ 1.77	594.25 $\pm$ 2.98**
DM + BAC (2)	236.79 $\pm$ 2.86	254.30 $\pm$ 3.28##	109.24 $\pm$ 2.58	289.90 $\pm$ 2.77##
DM + BAC (4)	246.65 $\pm$ 5.43	277.40 $\pm$ 5.78##	105.89 $\pm$ 1.69	245.37 $\pm$ 1.98##

\*\* $P < 0.01$  compared with Con group; ## $P < 0.01$  compared with DM group. Con: control; DM: diabetes; DM + BAC (2), baicalein (2 mg/kg)-treated; DM + BAC (4), baicalein (4 mg/kg)-treated groups.

tions [18]. In brief, we acquired three minute-adaptation and aversion trials in the apparatus during the training sessions. Following the training sessions, the avoidance of the dark shock-associated compartment and the latency to enter the dark compartment were measured after 24 h and 48 h. A latency of 3 min reflected that the animals did not enter the dark compartment.

### *Detections of acetylcholinesterase (AChE) and choline acetylase (ChAT) Activities in the hippocampus of STZ-induced diabetic rats*

After treatment with BAC or vehicle, the rats were anesthetized with were sacrificed immediately and the hippocampal samples for each group were dissected carefully. The activities of AChE and ChAT were measured with the corresponding commercial kits (kit No. A023 for AChE and kit No. A079-1 for ChAT, Nanjing Jiancheng Biotechnology Institute, Nanjing, China).

### *Western blot assay*

Western blot analysis were conducted as previously described [19]. For short, the hippocampal samples were homogenized in an ice-cold lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Then the lysates were centrifuged at 13,200  $\times$  g for 20 min at 4°C. The supernatant were acquired and the protein concentration were quantified by BCA protein assay kit (Beyotime Institute of Biotechnology, Nantong, China). Subsequently, 30  $\mu$ g protein were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes (Millipore, MA). The membranes were blocked with 5% non-fat milk

for 1 h at room temperature. Blots were incubated with the following primary antibodies: rabbit anti-phospho-PI3K (p-PI-3K, 1:1000; Cell Signaling Technology, USA), rabbit anti-phospho-Akt (p-Akt, 1:1000; Cell Signaling Technology, USA), rabbit anti-phospho-GSK3 $\beta$  (p-GSK3 $\beta$ ,

1:1000; Cell Signaling Technology, USA) and GAPDH (1:2000, Kang Chen, China), respectively, overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated second antibody (1:5000, Santa Cruz, USA) for 2 h. The immunodetection of protein bands was performed by an enhanced chemiluminescence kit (Pierce, CA). The immunoblots were subjected to the grey value analysis using Quantity One software.

### *Measurement of and caspase-9 and caspase-3 activities in the hippocampus of STZ-induced diabetic rats*

Caspase-9 and caspase-3 were regarded as executioner molecules in the apoptotic cascades. The amounts of caspase-9 and caspase-3 was measured at 405 nm following manufacturer's protocols (R & D Systems, USA).

### *Statistical analysis*

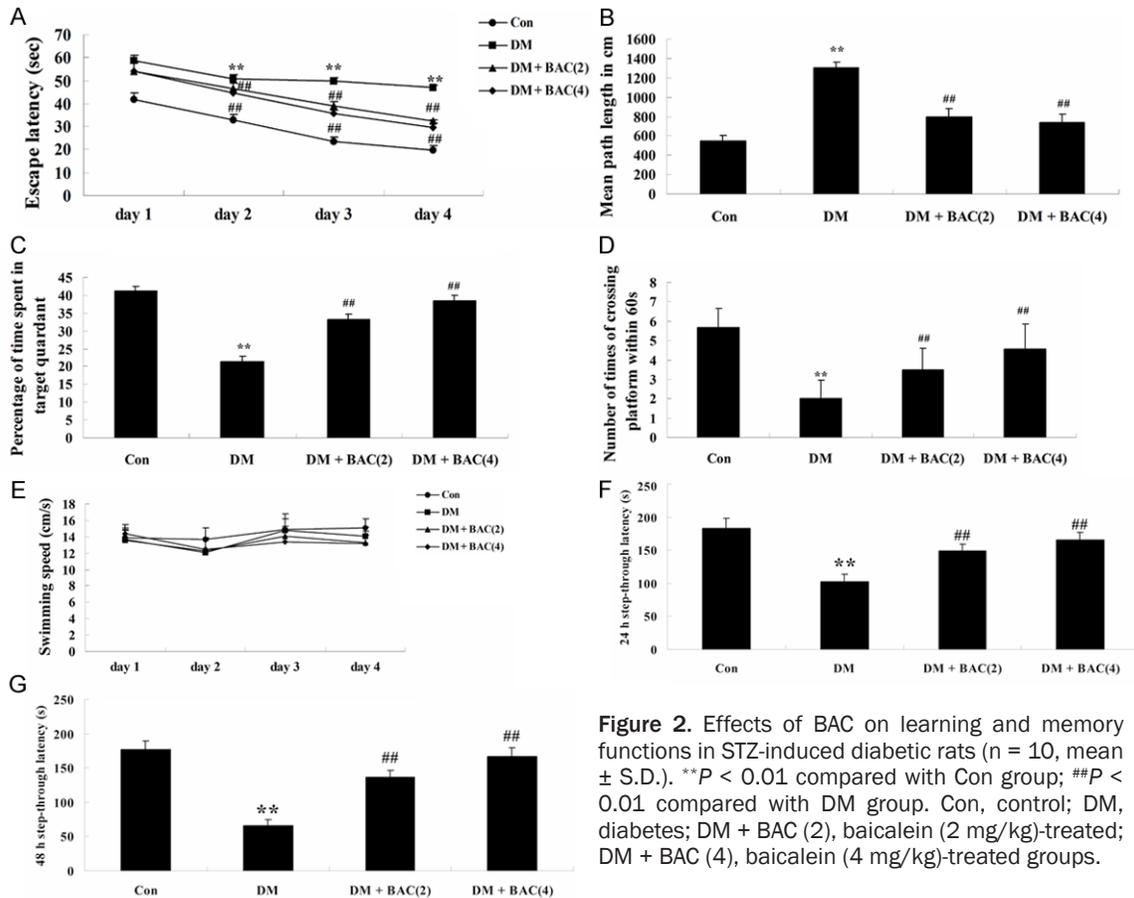
All values were given as mean  $\pm$  S.D. Statistical analysis was conducted using one-way ANOVA followed by Dunnett's test, with  $P < 0.05$  as the significant level.

## Results

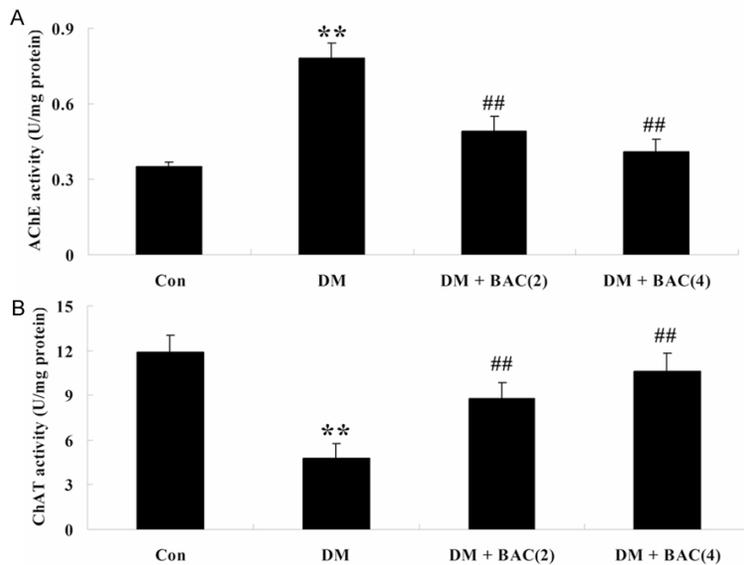
### *BAC influenced body weight and blood glucose levels in STZ-induced diabetic rats*

The chemical structure of BAC was displayed in **Figure 1**. **Table 1** indicated a marked reduction in the body weights of STZ-treated rats compared to the age-matched control group ( $P < 0.01$ ). Besides, diabetic rats exhibited significantly increased (594.25  $\pm$  2.98) plasma glucose levels than those of control group (113.45  $\pm$  1.77). BAC supplement by the doses of 2 mg/kg and 4 mg/kg for seven weeks could remark-

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**Figure 2.** Effects of BAC on learning and memory functions in STZ-induced diabetic rats (n = 10, mean  $\pm$  S.D.). \*\* $P < 0.01$  compared with Con group; ## $P < 0.01$  compared with DM group. Con, control; DM, diabetes; DM + BAC (2), baicalein (2 mg/kg)-treated; DM + BAC (4), baicalein (4 mg/kg)-treated groups.



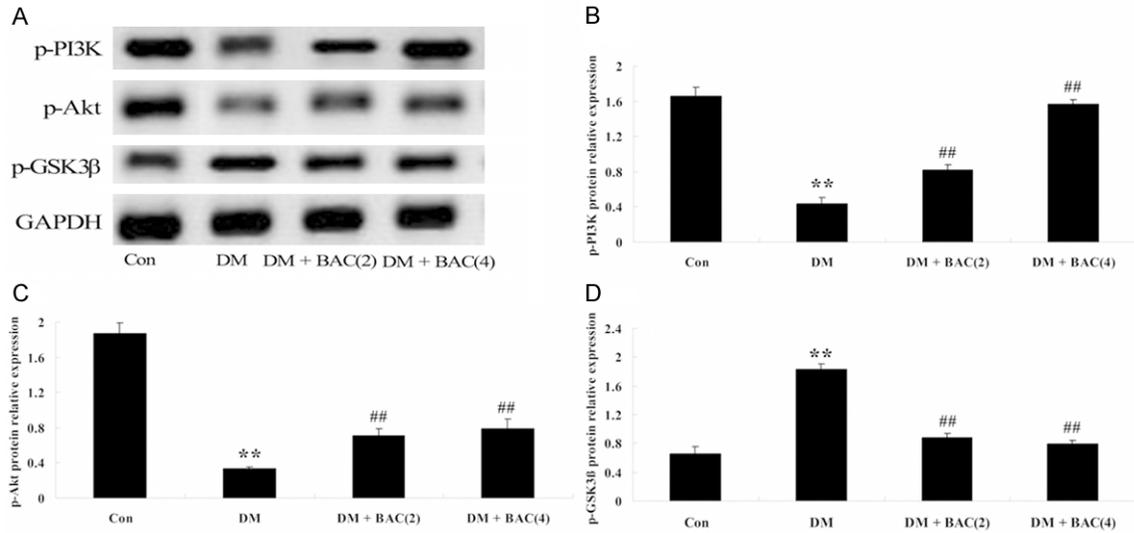
**Figure 3.** Effects of BAC on the activities of AChE and ChAT in diabetic rats hippocampus (n = 10, mean  $\pm$  S.D.). \*\* $P < 0.01$  compared with Con group; ## $P < 0.01$  compared with DM group. Con, control; DM, diabetes; DM + BAC (2), baicalein (2 mg/kg)-treated; DM + BAC (4), baicalein (4 mg/kg)-treated groups.

ably reverse the blood glucose levels and body weights in diabetic rats ( $P < 0.01$ ).

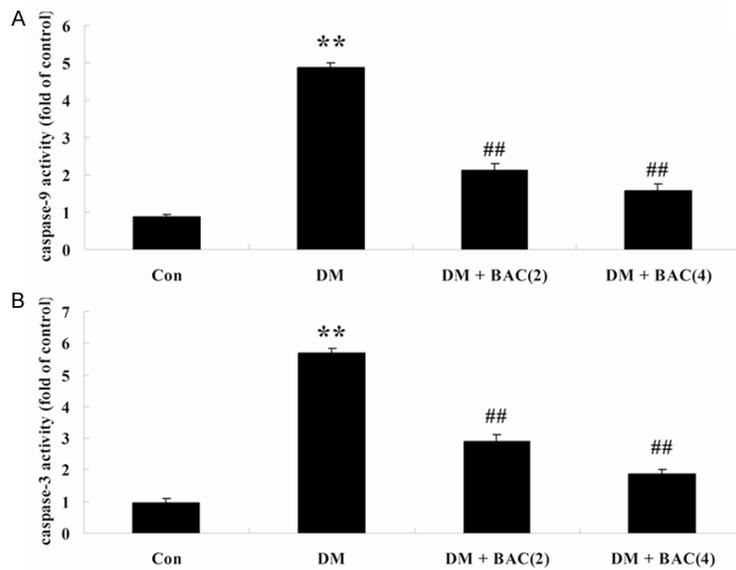
*BAC treatment improved learning and memory functions in STZ-induced diabetic rats*

The cognitive performance was evaluated in the Morris water maze test (7th week). There was a significant reduction of the mean escape latency from 60 to 20 s over the course of the 20 learning trials. Although no significant difference was found between any of the groups on the first day of testing in Morris water maze, the transfer latency was obviously different between diabetic ( $50.90 \pm 1.60$ ) and control rats ( $32.70 \pm 2.50$ ) ( $P$

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**Figure 4.** Effects of BAC on the protein levels of PI3K/Akt/GSK3 $\beta$  in diabetic rats hippocampus ( $n = 10$ , mean  $\pm$  S.D.). \*\* $P < 0.01$  compared with Con group; ## $P < 0.01$  compared with DM group. Con, control; DM, diabetes; DM + BAC (2), baicalein (2 mg/kg)-treated; DM + BAC (4), baicalein (4 mg/kg)-treated groups.



**Figure 5.** Effects of BAC on caspase-9 and caspase-3 activities in diabetic rats hippocampus ( $n = 10$ , mean  $\pm$  S.D.). \*\* $P < 0.01$  compared with Con group; ## $P < 0.01$  compared with DM group. Con, control; DM, diabetes; DM + BAC (2), baicalein (2 mg/kg)-treated; DM + BAC (4), baicalein (4 mg/kg)-treated groups.

$< 0.01$ ) from the 2nd day onwards. Chronic treatment with BAC (2 and 4 mg/kg) to diabetic rats dramatically decreased the mean escapes latency ( $P < 0.01$ ) (Figure 2A). Figure 2B also revealed a significant increase in mean path length in diabetic group for four consecutive training days compared with controls ( $P < 0.01$ ).

Nevertheless, BAC treatment at different doses markedly reduced this value when compared with the vehicle-treated diabetes ( $P < 0.01$ ). The probe trial was subsequently performed to explore how well the animals had learned and consolidated the platform location. Figure 2C showed there was an evident decline for the time spent in the target quadrant in diabetic group compared with controls ( $P < 0.01$ ). However, BAC (2 and 4 mg/kg) dramatically spent more time ( $P < 0.01$ ) in the target quadrant during the probe trial. Likewise, the number of times the animals crossed the former platform location was also decreased in diabetic rats ( $P < 0.01$ ) than those in control group, as indicated in

Figure 2D. But this index was significantly improved in diabetic group after administration of BAC (2 and 4 mg/kg) ( $P < 0.01$ ). In terms of swimming speed, there was no significant difference in swimming speed among different groups throughout the four training days (Figure 2E). Furthermore, the passive avoidance test

disclosed that there were marked reductions ( $P < 0.01$ ) of 24 h and 48 h step-through latencies in rats subjected to diabetes, in comparison to control group. When treatment with BAC at the doses of 2 and 4 mg/kg, these indices mentioned above were both reversed ( $P < 0.01$ ) in diabetic rats (**Figure 2F** and **2G**).

### *Effects of BAC on the activities of AChE and ChAT in diabetic rats hippocampus*

To figure out whether BAC could influence the activities of AChE and ChAT in diabetic rats hippocampus, colorimetric tests were performed in our current work. **Figure 3A** revealed that there was a marked elevation of AChE activity in the diabetic group ( $P < 0.01$ ), as compared to the controls. This value was remarkably prevented after BAC treatment. Conversely, the level of ChAT was found to be significantly decreased ( $P < 0.01$ ) in diabetic rats hippocampus and BAC treatment obviously elevated the ChAT activity in a dose-dependent manner (**Figure 3B**).

### *Effects of BAC on the protein levels of PI3K/Akt/GSK3 $\beta$ in diabetic rats hippocampus*

Western blot analysis were further conducted to explore whether BAC protected against DACD via PI3K/Akt/GSK3 $\beta$  signaling pathway in our present study. **Figure 4A** displayed the representative immunoblots with p-PI3K, p-Akt, p-GSK3 $\beta$  and GAPDH, respectively, from different groups. Quantitative analysis revealed that diabetic rats exhibited obviously reduced phosphorylation of PI3K and Akt while the evident elevation of p-GSK3 $\beta$  was observed ( $P < 0.01$ ), compared to the controls. Nonetheless, it was noteworthy that administration of BAC dose-dependently stimulated the levels of p-PI3K and p-Akt and suppressed GSK3 $\beta$  phosphorylation in diabetic group ( $P < 0.01$ ), compared with vehicle-treated group (**Figure 4B, 4D**).

### *Effects of BAC on the activities of caspase-9 and caspase-3 in diabetic rats hippocampus*

Caspase-9 and caspase-3 activities were both shown to be significantly elevated in hippocampus of diabetic animals after seven weeks ( $P < 0.01$ ), as illustrated in **Figure 5A, 5B**. However, BAC treatment (2 and 4 mg/kg) drastically inhibited ( $P < 0.01$ ) the activities of caspase-9 and caspase-3 in rats with diabetes.

## Discussion

The major findings of our current investigation showed that BAC significantly caused the decreases of blood glucose level, ChAT activity, caspase-9, caspase-3, p-PI3K and p-Akt accompanied with the improvement of body weight, learning and memory functions (by Morris water maze test and avoidance passive test), AChE activity and p-GSK3 $\beta$  in STZ-induced diabetic rats. These results implicate that BAC exerts a neuroprotective potential against DACD and its protection may be associated with altering PI3K/Akt/GSK3 $\beta$  signaling pathway.

Diabetes-induced cognitive impairment has the characteristics of learning and memory dysfunction, structure abnormality and neuronal damage [20]. A previous study reported the decline of cognitive efficiency over time in diabetic adults [21], indicating the correlation between diabetes and cognitive deficits. Meanwhile, pharmacological interference by some reagents could remarkably attenuated diabetes-induced learning and memory deficits [3-5]. Our present investigation also illustrated that BAC treatment significantly protected against DACD in rats. In fact, prior work depicted the amelioration of cognitive decline in epilepsy by BAC treatment [19], which was partly consistent with our current results.

The cholinergic neuron function is known to have a crucial role in learning and memory performance in mammalian limbic system and ACh is a key factor [22]. AChE and ChAT are two major enzymes that are highly expressed in cholinergic neurons. Under physical conditions, the stable concentration of ACh in cholinergic system are maintained by the balanced regulations of AChE and ChAT. AChE is responsible for the hydrolyzate of ACh while ChAT serves as the biosynthesis of ACh. It was reported that AChE and ChAT dynamically modulated the concentration of ACh in Alzheimer's disease [23], suggesting the critical role in learning and memory function. Our present investigation illustrated that AChE was observed to be evidently increased while the decreased ChAT was found in diabetic rats, which were in line with the previous findings. Nevertheless, this phenomenon was prevented after the administration of BAC to the diabetic rats.

Many studies strongly supported that PI3K/AKT/GSK3 $\beta$  signaling pathway plays a critical role in learning and memory processes [24, 25]. In detail, PI3K activation was previously reported to be involved in the improved spatial learning and memory ability of Purple sweet potato color against brain aging induced by D-galactose in old mice [25] and it was also found that activated PI3K is imperative for the activation and phosphorylation of Akt. What's more, the activations of PI3K and Akt was also observed to exacerbate the cognitive impairment caused by chronic cerebral hypoperfusion in rats [26]. In terms of GSK3 $\beta$ , it was previously demonstrated that the activated GSK3 $\beta$  was correlated with cognition-deficient cell model caused by A $\beta$  peptides [8]. Inhibition of GSK3 $\beta$  was found to ameliorate spatial learning and memory deficits in transgenic mice with Alzheimer's disease [9]. Our current work revealed that BAC augmented the phosphorylations of PI3K and Akt and decreased the phosphorylated level of GSK3 $\beta$  in diabetic group. A previous investigation demonstrated that BAC protected against neuronal excitotoxicity via activating PI3K/Akt pathway [27], which was consistent with our present data.

Caspases are specifically activated in response to apoptotic cascades and caspase-9 and caspase-3 are conceived of as the key executioners during apoptosis. Our present study revealed marked elevation of caspase-9 and caspase-3 activities in hippocampus of diabetic rats and this effect was inhibited by BAC treatment in a dose-dependent manner, suggesting that BAC reduced neuronal death in a diabetic rat model. A previous work demonstrated BAC antagonized rotenone-induced apoptosis in dopaminergic SH-SY5Y cells, which was in good agreement with our present results [28].

In summary, our novel findings disclosed that BAC exerted beneficial effects against DACD in rats and its neuroprotection might be linked with activating PI3K and Akt phosphorylation accompanied with suppressing the phosphorylated level of GSK3 $\beta$ . These results hint that BAC is likely to be served as an adjuvant therapy to conventional anti-hyperglycemic regimens as well as DACD. However, further investigation is required to verify this research.

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### Disclosure of conflict of interest

None.

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