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Original Article

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Dihydromyricetin improves type 2 diabetes-induced cognitive impairment via suppressing oxidative stress and enhancing brain-derived neurotrophic factor-mediated neuroprotection in mice

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Abstract

Type 2 diabetes mellitus (T2DM) leads to cognitive impairment (CI), but there have been no effective pharmacotherapies or drugs for cognitive dysfunction in T2DM. Dihydromyricetin (DHM) is a natural flavonoid compound extracted from the leaves of *Ampelopsis grossedentata* and has various pharmacological effects including anti-oxidant and anti-diabetes. Thus, we investigated the effects of DHM on CI in T2DM mouse model and its possible mechanism. To induce T2DM, mice were fed with high-sugar and high-fat diet for 8 weeks, followed by a low dose streptozotocin (STZ) administration. After the successful induction of T2DM mouse model, mice were treated respectively with equal volume of saline (T2DM group), 125 mg/kg/d DHM (L-DHM group), or 250 mg/kg/d DHM (H-DHM group). After 16 weeks of DHM administration, the body weight (BW), fasting blood glucose, blood lipids, intraperitoneal glucose tolerance (IPGT), and cognitive function were determined. Then, alterations in the expressions of oxidative stress markers and brain-derived neurotrophic factor (BDNF) in the hippocampus were investigated. Our findings demonstrated that DHM could significantly ameliorate CI and reverse aberrant glucose and lipid metabolism in T2DM mice, likely through the suppression of oxidative stress and enhancement of BDNF-mediated neuroprotection. In conclusion, our results suggest that DHM is a promising candidate for the treatment of T2DM-induced cognitive dysfunction.

Key words: dihydromyricetin, T2DM, cognitive impairment, oxidative stress, brain-derived neurotrophic factor

Introduction

Accumulating evidence demonstrates that type 2 diabetes mellitus (T2DM) leads to cognitive impairment (CI) [1,2]. Patients with diabetes mellitus (DM) are more likely to suffer from Alzheimer's disease characterized by cognitive dysfunction [3]. However, there has been no effective pharmacotherapy for T2DM-induced cognitive dysfunction. Although the exact mechanism of T2DM-induced cognitive dysfunction remains unknown, hyperglycemia, abnormal insulin signaling, and oxidative stress have been considered as the main factors in its pathogenesis [4].

Diabetes-induced oxidative stress results in oxidative damage in different regions of the brain, leading to CI [5]. Anti-oxidant therapy has been shown to protect neurons against diabetes-induced oxidative insults [6].

Brain-derived neurotrophic factor (BDNF), a neuroprotective protein in the brain, is crucial in the development of central nervous system, particularly in neuronal survival and synapse formation [7]. It was reported that plasma BDNF levels were obviously decreased in patients with T2DM [8]. Recent studies showed that decreased levels of BDNF in the hippocampus were associated with impaired cognition in streptozotocin (STZ)-induced diabetic rats [9,10], suggesting that BDNF is closely related to the cognitive dysfunction in diabetes. Moreover, BDNF has been demonstrated to regulate glucose metabolism in the pancreas of diabetic mice [11], and the insulin signaling can be rapidly enhanced after the administration of BDNF [12]. These studies suggest that BDNF may play a pivotal role in modulating cognitive dysfunction in T2DM.

Dihydromyricetin (DHM) is a natural flavonoid compound extracted from the leaves of *Ampelopsis grossedentata* (**Fig. 1A**) [13]. Many studies have shown that DHM has several pharmacological functions, such as anti-inflammation, anti-oxidation, and positive effect in diabetic treatment [13–15]. Recently, DHM was shown to improve behavioral deficits and reverse neuropathology in transgenic mouse models of Alzheimer's disease [16]. All these findings suggest that DHM can potentially be used to treat T2DM-induced cognitive dysfunction.

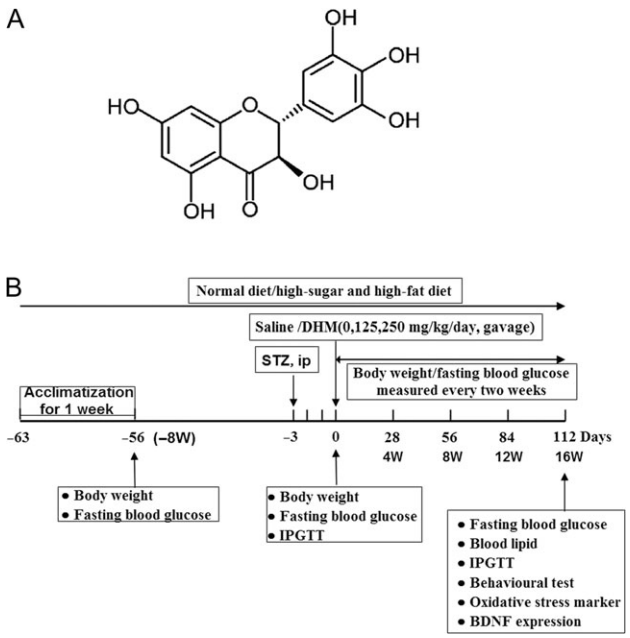

Figure 1. Chemical structure of DHM (A) and experimental scheme (B)

Thus, in the present study, we aimed to investigate the ameliorative effects of DHM on CI in T2DM mouse model and its possible mechanism. The behavioral alterations of T2DM mice after DHM treatment were first measured, including locomotor activities and Morris water maze (MWM) tests. We also determined the changes in body weight, blood glucose content, the levels of lipids metabolism and oxidative stress markers as well as the expression levels of BDNF in the hippocampus. Our results showed that DHM significantly ameliorated metabolic and cognitive dysfunction in T2DM mice. These therapeutic effects may result from the suppression of oxidative stress and BDNF-mediated neuroprotection.

Materials and Methods

Materials

DHM (purity ≥98%) was purchased from Zhicheng Biotechnology Company (Zhangjiajie, China). STZ was purchased from Sigma-Aldrich (St Louis, USA). Oxidative stress assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rabbit polyclonal anti-BDNF antibody was purchased from Cell Signaling Technology (Beverly, USA) and mouse monoclonal anti-β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). TRIzol reagent and SYBR Green I dye were purchased from Invitrogen (Carlsbad, USA).

Experimental animals

Totally, 40 male C57BL/6J mice (4 weeks old, 18 ± 2 g body weight) were purchased from the Hunan SLAC Laboratory Animal Co., Ltd (Changsha, China). The mice were maintained under a regular 12-h light period at a controlled temperature (22 ± 2°C) and received chow and water *ad libitum*. Animal care and treatments were conducted according to established guidelines and protocols approved by the Animal Care and Use Committee of the University of South China (Hengyang, China). All efforts were made to minimize the number of animals used and their sufferings.

Induction of type 2 diabetic mouse model

After 1 week of acclimatization, mice were randomly divided into normal control group (n = 8) and T2DM group (n = 32). Mice in the normal control group were fed with normal diet. Type 2 diabetes model was induced as previously described [17–20] with slight modifications. In brief, mice were fed with a high-sugar and high-fat diet (HSF; formula: 15% sucrose, 10% lard, 5% egg yolk powder, 1% cholesterol, 1% sodium chloride, and 68% standard rat feed) for 8 weeks. After being fasted for 12 h, mice received an intraperitoneal (i.p.) injection of STZ at a dose of 100 mg/kg [17,18] for 3 days. At the end of STZ injection, five mice died and were excluded from the experiment. Meanwhile, mice in the control group received injection of citrate buffer solution at the same dosage (i.p.). After 3 days of STZ injection, IPGT and FBG were determined with a blood glucose monitoring sensor (Accu-Chek; Roche, Shanghai, China) to check the successful establishment of T2DM mouse model. Only mice with glucose level ≥11.1 mM were regarded as successful T2DM model mice and used in the subsequent experiments (three mice were removed from the T2DM group).

Experimental scheme

The experimental scheme of the present study was illustrated in **Fig. 1B**. T2DM model mice (n = 24) were randomly divided into three groups: T2DM, L-DHM, and H-DHM group. Mice were fed with HSF and, respectively, treated with an equal volume of saline

or DHM (at the dosage of 125 or 250 mg/kg/d) by gavage for 16 weeks. Mice in the normal control group ($n = 8$) were fed with normal diet and daily treated with equal volume of saline for 16 weeks. Then, the body weight (BW) and fasting blood glucose (FBG) were determined. Subsequently, mice were subject to the Y-maze and MWM tests to assess spatial learning and working memory. After behavioral tests, mice were immediately sacrificed and brain tissues were collected for further examination.

Y-maze test

The Y-maze test is a simple two-trial recognition test for measuring spatial working memory. The apparatus was made of mahogany-painted wood with three arms placed symmetrically at a 120° from one another. The walls were 15 cm high and the arms were 8 cm wide and 30 cm long. Each arm had special cues on the walls so that the mice could discriminate the distinction of arms from each other. To avoid the disturbance of odors, the maze arms were thoroughly cleaned during the interval of two tests. Each mouse was placed at the end of one arm and allowed to freely explore in the three arms for 8 min. The number of arm entries was manually recorded. Complete entry including the base of the animal's tail was regarded as one successful arm entry. Alternation was defined as a consecutive entry in three different arms through the overlapping triplet sets [21,22]. The alternation percentage was calculated with the following formula: $SA\% = n/(N-2) \times 100\%$, in which n is the number of alternations, and N is the total number of arm visits.

Morris water maze test

The MWM test was conducted as described previously with slight modifications [23,24]. Briefly, mice were individually trained in a circular water pool (120 cm in diameter and 50 cm high) filled with water (22°C ± 2°C). The pool was artificially divided into four quadrants (W, E, S, and N zones), labeled with different visual cues. A platform (6 cm in diameter) was randomly placed in the center of one quadrant and its location was fixed during the training sessions. Each trial was started in the pseudo-randomized points in different quadrant with the animals facing toward the wall. All mice were individually trained in both of the visible-platform (Day 1) and hidden-platform (Days 2–5) phases. The visible platform training was performed to determine the baseline difference of vision, motivation, and motor function in different treatment groups. The platform was placed 1 cm below the water surface and was indicated by a small red flag (5 cm in height). The hidden-platform training phase was conducted to evaluate spatial learning ability. During this phase, the platform was placed 1 cm below the water surface without indicator and its location was fixed. Each animal was subject to four trials a day with 1 h interval. Each trial examined the ability of the mouse to reach the platform within 90 s. If the animal failed to find the platform within 90 s, it will be gently guided to the platform for 30 s. On Day 6, the platform was removed and mice were subject to one probe trial, in which animals were required to search for the platform within 90 s. The time that the animal stayed in the target quadrant and the number that it crossed over the location, where the former platform was in, was recorded. The probe trail can be used to assess the retention of spatial memory. Data such as the latency to reach the platform (escape latency), swimming distance, and the percentage of time and the number of entries in the target quadrant were collected by the video tracking equipment and analyzed by the analysis-management system (Viewer 2 Tracking Software; Chengdu Techman Software Company, Chengdu, China).

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance test (IPGTT) was conducted after an overnight fasting. Mice were injected with glucose dissolved in distilled water (100 mg/ml) at the dose of 1 g/kg (i.p.). Then, blood glucose in tail blood samples collected at 0 (prior to glucose administration), 15, 30, 60, 90, and 120 min post-glucose administration was quantified.

Determination of FBG and blood lipids

Mice were fasted for 12 h and then anesthetized with sodium pentobarbital (40 mg/kg, i.p.). Blood samples were collected from the inner canthus vein of the eye into plastic centrifuge tubes without anti-coagulant. Then, the tubes were centrifuged at 999 *g* for 10 min at 4°C. The plasma was separated and loaded into an HI-TACH717 automatic biochemical analyzer (Thermo, Waltham, USA) to determine the levels of FBG, TG, TC, HDL, and LDL cholesterol.

Measurement of oxidative stress markers in the hippocampus

Hippocampus tissues were homogenized in lysis buffer and the supernatant was used for the measurement of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-PX) using commercial assay kits, respectively (S0131, S0109, S0051, and S0056; Beyotime Institute of Biotechnology, Shanghai, China). The concentration of MDA, which is an indicator of lipid peroxidation, was measured using the thiobarbituric acid (TBA) method with MDA Assay kit. Briefly, 0.1 ml of the hippocampus tissue supernatant was mixed with 200 µl MDA working solution, heated in a heat block (100°C) for 15 min, and then cooled down to room temperature. After centrifugation at 1000 *g* for 10 min, the supernatant was measured at a wavelength of 532 nm, and MDA level unit was expressed as nmol/mg of protein. The assay for SOD activity was based on the ability of SOD to inhibit nitroblue tetrazolium (NBT) reduction by superoxide. Briefly, 20 µl of the tissue supernatant was mixed with 160 µl NBT working solution and 20 µl reacting solution and then incubated in 37°C for 30 min. Finally, the optical density was measured at a wavelength of 560 nm. The amount of protein that inhibited NBT reduction to 50% of the maximum was defined as 1 nitrite unit (NU) of SOD activity. The CAT activity was detected using Catalase Analysis Kit according to the manufacturer's instructions. Briefly, the tissue supernatant was treated with excess hydrogen peroxide (H_2O_2) for decomposition for an indicated time. The remaining H_2O_2 coupled with a substrate was treated with peroxidase to produce N-4-antipyryl-3-chloro-5-sulfonate-*p*-benzoquinonemonoimine, which absorbs maximally at a wavelength of 520 nm. One unit (U) of CAT activity is defined as the amount of enzyme catalyzing 1 µmol of H_2O_2 per mg per min at 25°C. GSH-PX activity measurement was performed in triple using GSH-PX assay kit. Briefly, 10 µl of the hippocampus tissue supernatant was mixed with 176 µl of GSH-PX buffer solution, 10 µl of GSH-PX working solution and 4 µl of 15 mM H_2O_2, then the mixture was measured at a wavelength of 340 nm. One unit of GSH-PX activity was defined as 1 µmol NADPH oxidized per min at pH 8.0 and 25°C.

Quantitative real-time PCR analysis

To analyze BDNF mRNA expression, quantitative real-time PCR (qRT-PCR) was performed [25]. Total RNA was extracted from hippocampus samples using Trizol reagent (Beyotime) according to

the manufacturer's instructions. The mRNA was quantified using SYBR green PCR master mix and a Light Cycler Real Time PCR system (Bio-Rad, Hercules, USA). The primers were synthesized by Sangon Biotech Engineering Co., Ltd (Shanghai, China) with the following sequences: (i) *BDNF* forward 5′-ATGGGTTACACGAA GGAAGG-3′ and reverse 5′-CCGAACATACGATTGGGTAGT-3′ (84 bp); and (ii) *β-actin* forward 5′-AGGCCCCTCTGAACCCTAA G-3′ and reverse 5′-CCAGAGGCATACAGGGACAAC-3′ (118 bp). The PCR conditions were 94°C for 4 min, 32 cycles of 94°C for 60 s, 62°C for 30 s and 72°C for 60 s, followed by a 72°C extension for 10 min. The relative expression of BDNF was calculated using the $2^{-\Delta\Delta Ct}$ method [26].

Western blot analysis

The hippocampus was homogenized in ice-cold lysis buffer (0.125 M Tris-HCl, pH 6.8, 0.2 M DTT, 4% SDS, and 20% glycerol). The lysates were sonicated for 10 min and centrifuged at 11,100 *g* (4°C) for 5 min to remove insoluble debris, and the protein concentration was determined using a BCA™ protein assay kit (Thermo Scientific, Rockford, USA). Total proteins (30 μg) were separated by SDS-PAGE (12%) and transferred onto a PVDF membrane (Millipore, Billerica, USA). After being blocked with 5% defatted milk for 1 h at room temperature, the membranes were incubated at 4°C overnight with anti-BDNF and anti-β-actin (loading control, 1:1000) primary antibodies. Membranes were then washed three times with TBST and then incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000) for 2 h at room temperature. Membranes were washed again and protein bands were detected using an enhanced chemiluminescence (ECL) kit (Beyotime).

Statistical analysis

The data were expressed as the mean ± SEM. The statistical significance was determined using one-way or two-way ANOVA followed by Fisher's LSD multiple comparisons test. $P < 0.05$ was considered of significant difference. To provide a detailed explanation for the effects of DHM on cognition, covariance analysis was performed to exclude the influence of BW, FBG, TC, TG, HDL, and LDL. The data were analyzed using SPSS 17.0 software.

Results

Establishment of type 2 diabetic mouse model

As shown in **Fig. 2A**, the BW of mice in the control group was increased from 18.4 ± 1.3 to 23.5 ± 2.1 g with normal diet feeding, whereas mice in the T2DM group gained much more weight during 8 weeks of HSF feeding compared with the control group. The assessment of the FBG indicated that the blood glucose concentration was significantly elevated after HSF feeding and STZ administration (**Fig. 2B**). The IPGTT results suggest the plasma glucose peaked 30 min after intraperitoneal glucose administration in both the control and T2DM groups (**Fig. 2C**). However, the blood glucose concentration was significantly higher and the metabolism of glucose was obviously retarded, which could be defined as glucose intolerance in the T2DM group compared with the control group (**Fig. 2C**). These results indicated that the establishment of T2DM mouse model was successful.

DHM can improve spatial learning and working memory in T2DM mice

To determine whether DHM has therapeutic effects on CI induced by T2DM, behavioral tests were conducted to evaluate spatial learning

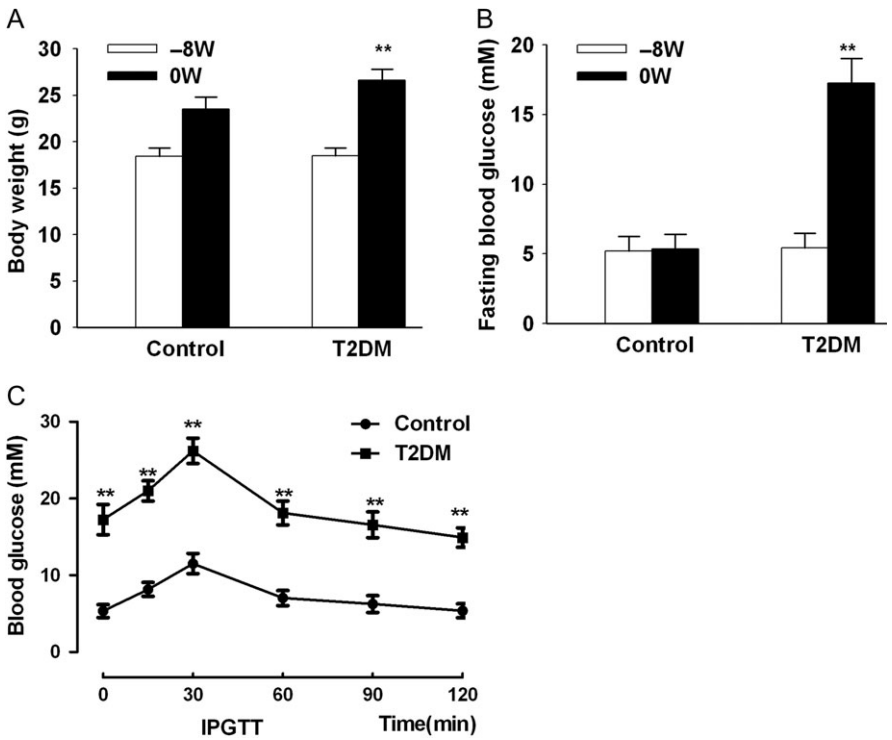

Figure 2. Altered levels of body weight (A), fasting blood glucose (B), and blood glucose (C) following IPGTT in mice Values are expressed as the mean ± SEM ($n = 5 \sim 8$). **Significant difference from respective controls at $P < 0.01$. Con: control group; T2DM: type 2 diabetes model group.

and memory. The Y-maze test was used to assess hippocampal-dependent spatial working memory. As shown in **Fig. 3A**, mice in the T2DM group demonstrated a significantly impaired spatial working memory compared with the control group, whereas mice treated with both dosages of DHM displayed an improved spontaneous alternation behavior. However, no statistical differences were found in the number of arm entries among all the experimental groups, suggesting that the locomotor activity was not influenced by T2DM (**Fig. 3B**).

Moreover, spatial learning and memory were examined by the MWM test. The escape latencies and swimming distances were consecutively recorded for 5 days. On the first day of visible-platform test, no significant difference was observed in the escape latency and swimming distance among the four groups, which excludes the possibility of motivational and sensory motor deficits induced by T2DM (**Fig. 4A,B**). Thereafter, a 4-day hidden-platform test was conducted. It was found that the escape latency of the T2DM group

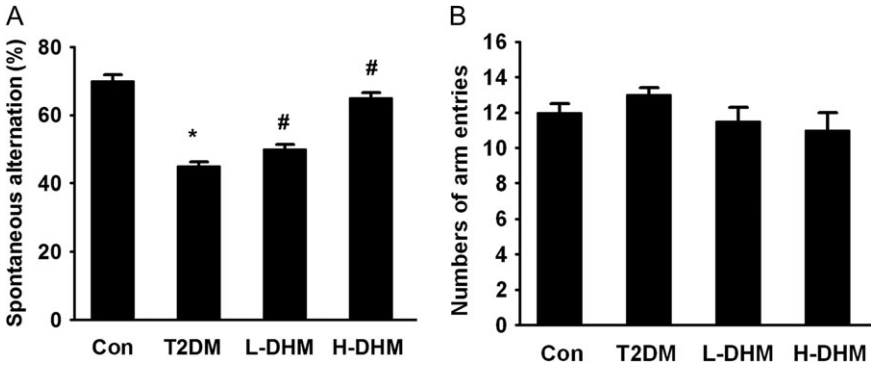

Figure 3. Effect of DHM on spontaneous alternation behavior Spontaneous alternation behavior (A), and number of arm entries/5 min (B). Values are expressed as the mean ± SEM ($n = 8$). $^{*}P < 0.05$ vs the Con group; $^{\#}P < 0.05$ vs T2DM group. Con: control group; T2DM: type 2 diabetes model group; L-DHM: 125 mg/kg/d DHM-treated T2DM group; H-DHM: 250 mg/kg/d DHM-treated T2DM group.

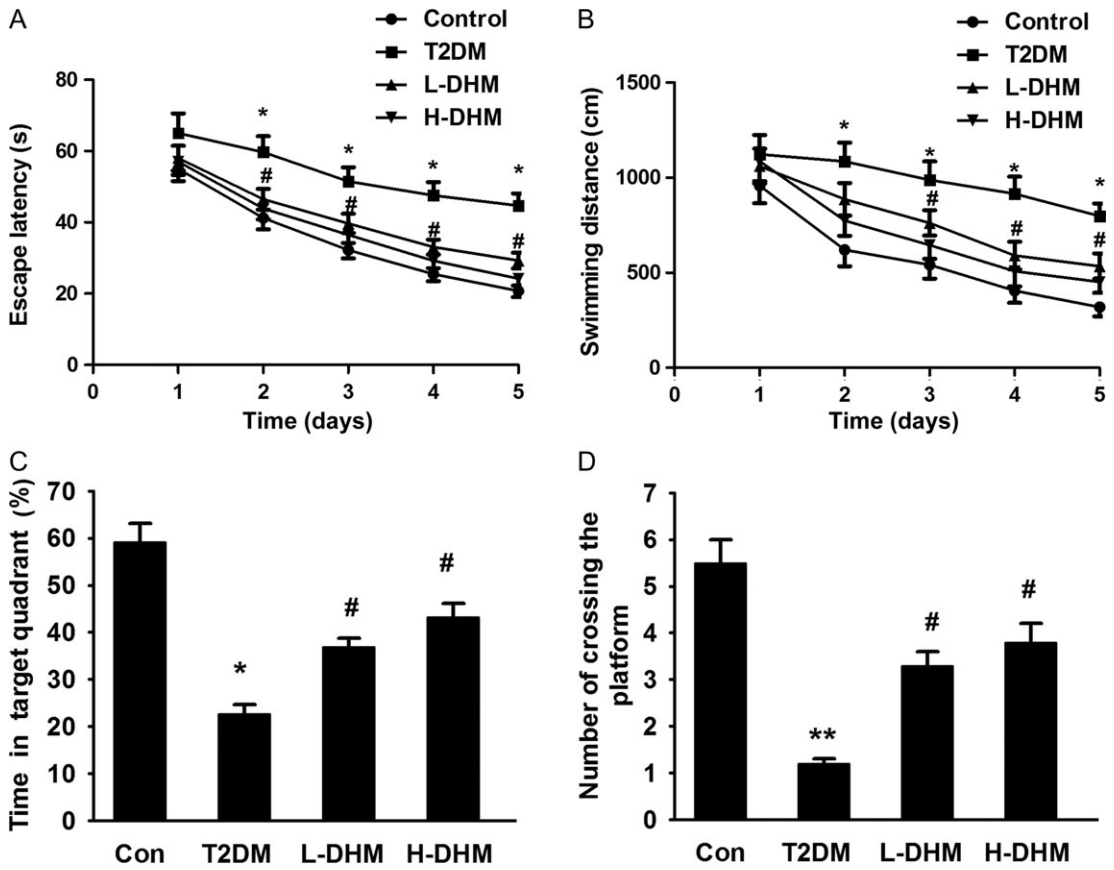

Figure 4. Effect of DHM on spatial learning and working memory in T2DM mice revealed by Morris water maze test Escape latency (A), swimming distance (B), the percentage of time in the target quadrant (C), and the number of crossing the platform (D). Values are expressed as the mean ± SEM ($n = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs the Con group; $^{\#}P < 0.05$ vs T2DM group. Con: control group; T2DM: type 2 diabetes model group; L-DHM: 125 mg/kg/d DHM-treated T2DM group; H-DHM: 250 mg/kg/d DHM-treated T2DM group.

Table 1. Effects of DHM on body weight, fasting blood glucose and blood lipid in T2DM mice

Variables	Control	T2DM	L-DHM	H-DHM
Final BW (g)	29.8 ± 1.1	25.9 ± 1.2*	27.6 ± 0.8$^{\#}$	28.5 ± 0.9$^{\#}$
Final FBG (mM)	5.32 ± 0.45	21.14 ± 1.92**	8.35 ± 1.81$^{\#\#}$	7.58 ± 2.63$^{\#\#}$
TC (mM)	2.02 ± 0.13	4.37 ± 0.21**	3.51 ± 0.18$^{\#\#}$	2.83 ± 0.16$^{\#\#}$
TG (mM)	0.92 ± 0.12	1.53 ± 0.14**	1.27 ± 0.09$^{\#\#}$	1.16 ± 0.11$^{\#\#}$
HDL (mM)	1.34 ± 0.09	0.93 ± 0.06**	1.09 ± 0.13$^{\#\#}$	1.21 ± 0.08$^{\#\#}$
LDL (mM)	0.43 ± 0.02	1.62 ± 0.13**	1.08 ± 0.09$^{\#\#}$	0.67 ± 0.05$^{\#\#}$

*$P < 0.05$, **$P < 0.01$ vs the Con group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs T2DM group.

was significantly longer than that of the control group during the test (**Fig. 4A**). Additionally, as shown in the **Fig. 4B**, mice in the T2DM group displayed a significantly longer swimming distance to find the platform. Our results did not show any significant reduction of the escape latency or swimming distance to find the platform during the training phases between two dosages (125 and 250 mg/kg/d, i.p.) of the DHM (**Fig. 4A,B**). One day after the training phase, mice received a probe test, in which the platform was removed. As shown in **Fig. 4C**, mice in the T2DM group displayed a significantly decreased duration in the target quadrant and a reduced target crossing number over the former location of the platform (**Fig. 4D**) compared with those of the control group. In contrast, treatment with either dosage (125 or 250 mg/kg/d, i.p.) of DHM reversed the decline in spatial memory of mice.

These data revealed that DHM treatment at the dosage of 125 or 250 mg/kg/d could significantly alleviate T2DM-induced deficits in spatial learning and working memory.

Effect of DHM on BW, FBG, blood lipid, and IPGTT in T2DM mice

The BW of normal mice is gradually increased over time, but the BW gain in mice with T2DM is stagnant. In our study, the final BW of the T2DM group was ~87% of that in the control group (**Table 1**). Furthermore, diabetic mice exhibited marked elevation in FBG level when compared with the control group. The BW of mice treated with DHM (125 and 250 mg/kg/d) for 16 weeks was significantly increased with a decreased FBG level (**Table 1**). Meanwhile, the blood concentrations of cholesterol (TC), triglyceride (TG), and low-density lipoprotein (LDL) were found to be significantly elevated and the level of high-density lipoprotein cholesterol (HDL) was significantly decreased in the T2DM group compared with the control group. DHM treatment at the dosage of either 125 or 250 mg/kg/d significantly reversed the alterations in the lipid profile. Moreover, the blood glucose level at 0 min was statistically higher in the T2DM group. The blood glucose peaked at 30 min after glucose loading in all the four groups. The blood glucose in the T2DM group maintained a high level during the IPGTT, whereas the blood glucose metabolism of DHM-treated groups (125 and 250 mg/kg/d) was faster than that of the T2DM group (**Fig. 5**), indicating that DHM treatment can alleviate the impaired glucose tolerance in T2DM mice.

DHM suppresses oxidative stress in the hippocampus of T2DM mice

To examine the effects of DHM on oxidative stress in the hippocampus, we measured the expression levels of malondialdehyde (MDA) and anti-oxidant enzymes, such as superoxide dismutase (SOD), catalase activity (CAT) and glutathione peroxidase (GSH-PX). As

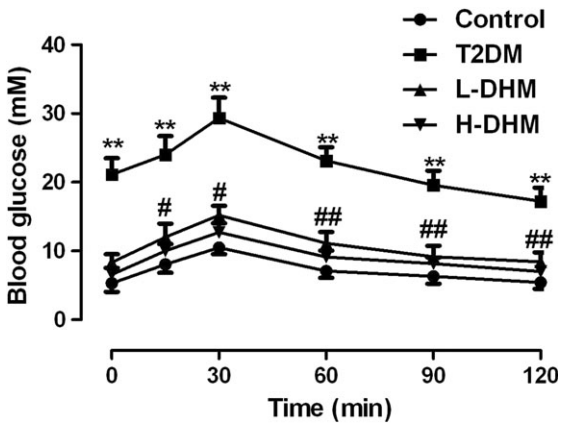

Figure 5. Effect of DHM on IPGT in T2DM mice Intraperitoneal glucose tolerance test (IPGTT) was performed. Values are expressed as the mean ± SEM ($n = 3$). **$P < 0.01$ vs the Con group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs T2DM group. Con: control group; T2DM: type 2 diabetes model group; L-DHM: 125 mg/kg/d DHM-treated T2DM group; H-DHM: 250 mg/kg/d DHM-treated T2DM group.

shown in **Fig. 6A**, the expression level of MDA was significantly increased in the T2DM group. Treatment with either dosage of DHM (125 and 250 mg/kg/d) significantly decreased the expression level of MDA in mice. Moreover, the activities of all the antioxidants including SOD, CAT, and GSH-PX were significantly decreased in the T2DM group compared with the control group. However, the activities of SOD, CAT, and GSH-PX were increased after DHM (125 and 250 mg/kg/d) treatment (**Fig. 6B–D**). These data indicated that DHM suppresses oxidative stress in the hippocampus of T2DM mice.

DHM increases the mRNA and protein expressions of BDNF in the hippocampus of T2DM mice

We further determined the underlying mechanisms by which DHM exerts its ameliorative effects on CI in T2DM mice. The mRNA and protein expressions of hippocampal BDNF were measured. As illustrated in **Fig. 7A,B**, the mRNA and protein expression levels of BDNF were significantly decreased in the T2DM group compared with the control group. However, DHM treatment resulted in a dose-dependent increase in BDNF mRNA and protein expression, which indicated that DHM increased the expression of BDNF in the hippocampus of T2DM mice.

Discussion

T2DM is a metabolic disorder with an increasing incidence, which affects a large number of people worldwide [27]. T2DM can cause

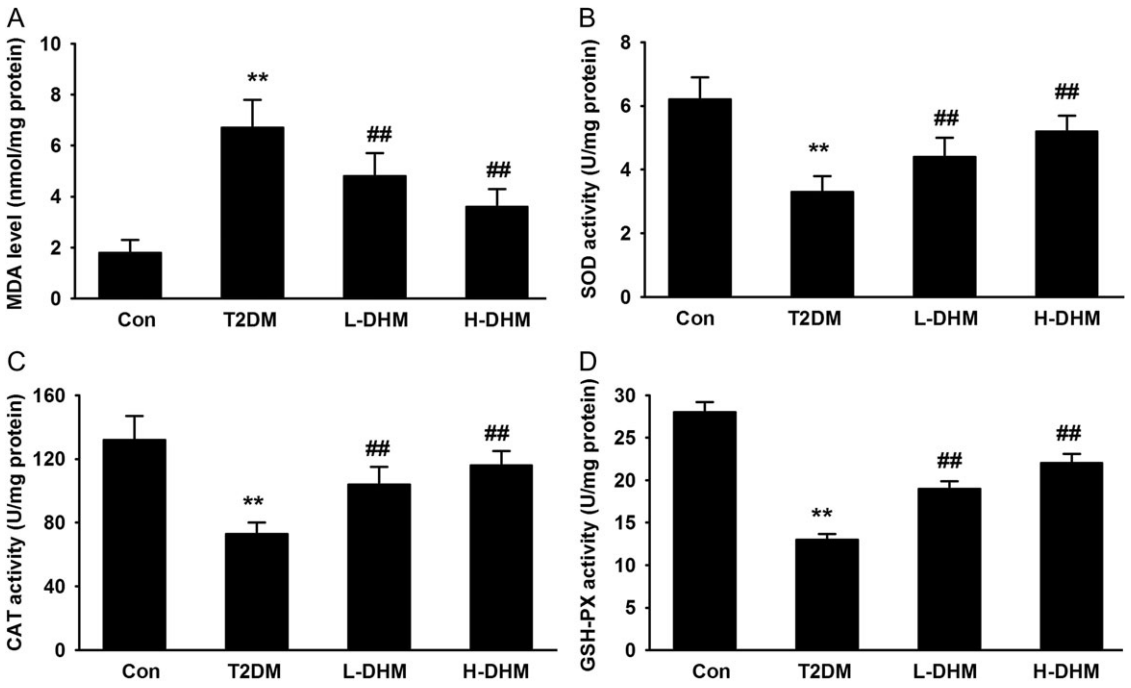

Figure 6. Effects of DHM on oxidative stress in the hippocampus of T2DM mice (A–D) Oxidative production of MDA, SOD, CAT, and GSH-PX activities, respectively in the hippocampus. Values are expressed as the mean ± SEM ($n = 5$). $^{**}P < 0.01$ vs the Con group; $^{\#\#}P < 0.01$ vs T2DM group. Con: control group; T2DM: type 2 diabetes model group; L-DHM: 125 mg/kg/d DHM-treated T2DM group; H-DHM: 250 mg/kg/d DHM-treated T2DM group.

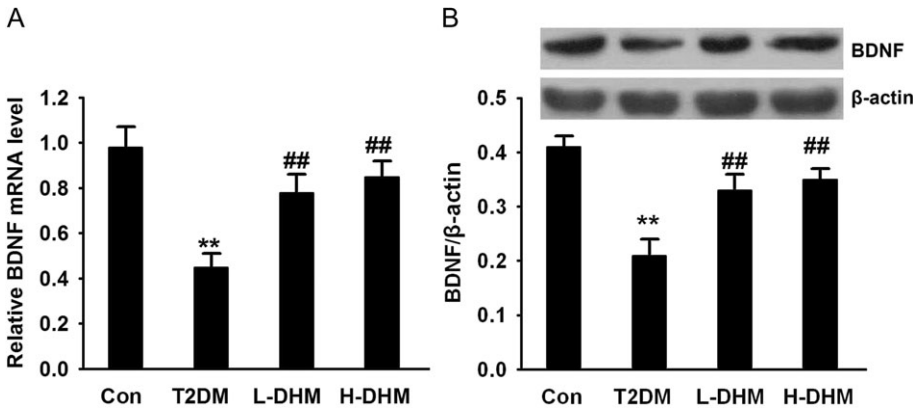

Figure 7. Effects of the DHM on the mRNA and protein expression levels of BDNF in the hippocampus (A) BDNF mRNA expression was measured by qRT-PCR, and relative mRNA level was calculated as the change relative to the control group level. (B) BDNF protein level was measured by western blot analysis. Representative immunoblots (top) and densitometric analysis (bottom) of the BDNF protein level normalized to internal β-actin level were presented. Data are the mean ± SEM ($n = 5$). $^{**}P < 0.01$ vs the Con group; $^{\#\#}P < 0.01$ vs T2DM group. Con: control group; T2DM: type 2 diabetes model group; L-DHM: 125 mg/kg/d DHM-treated T2DM group; H-DHM: 250 mg/kg/d DHM-treated T2DM group.

the damage to multiple systems, leading to complications. Accumulating studies have demonstrated that T2DM can result in cognitive dysfunction [28]. DHM is extracted from the grape plant rattan tea whose main active ingredient is dihydrogen flavonoid, and has been shown to have hypoglycemic, lipid-lowering, and many other pharmacological effects [29]. Particularly, DHM has the potential to treat cognitive dysfunction and neuropathological damage [30,31]. Thus, in the present study, we examined the therapeutic effects of DHM on T2DM-induced CI, and further determined its possible mechanism.

DM-induced CI is characterized by the decline in episodic, language, and spatial memory [32]. Consistently, mice in the T2DM group showed significantly impaired behavioral performance in both the Y-maze test and MWM test. DHM treatment can reverse T2DM-induced spatial learning and working memory impairment. These findings suggest that the restoration of behavioral function may be highly related to the neuroprotective effects after DHM treatment.

It has been well established that weight loss, hypercholesteremia, hyperlipidemia, and hyperglycemia are typical diabetic signs [33]. Currently, several factors, such as glucose metabolism disorder and its ensuing hyperglycemia [34,35], which increase oxidative stress reaction [36] and impair nerve growth factor and BDNF signaling [37,38], have been implicated in the pathogenesis of DM-induced

cognitive dysfunction. Persistent hyperglycemia is the major cause of most complications induced by diabetes. It has also been suggested that chronic hyperglycemia may directly result in the CI in diabetes, suggesting that glycemic control may contribute to the treatment of diabetic cognitive impairment (DACI). In our study, we observed that the BW of mice in the T2DM group was significantly higher than the control group after 8 weeks of HSF feeding. Meanwhile, the FBG level was obviously elevated in the T2DM group compared with the control group. In addition, the blood glucose level was significantly higher in the T2DM group than that in the control group during the IPGTT. These results suggested that the T2DM mouse model was successfully established. Subsequently, T2DM mice continued to receive HSF for 16 weeks. We noticed an impaired glucose tolerance, and the levels of FBG, TC, TG, and LDL were significantly increased, while the BW and HDL levels were significantly decreased in the T2DM group compared with the control group. However, treatment with DHM for 16 weeks markedly reversed T2DM-induced weight loss and dysfunctions in glucose and lipid metabolism in mice, suggesting a positive role of DHM in hyperglycemic therapy. Therefore, we speculate that the anti-hypercholesteremia and anti-hyperlipidemia effects of DHM might contribute to the alleviation of T2DM-induced CI.

Moreover, oxidative stress is closely related to the pathogenesis of T2DM. It can result in morphological and functional alterations in brain regions, leading to learning and memory deficits [36]. MDA is a specific product of lipid peroxidation in cell membranes in the process of free radical production [39,40]. Oxidative injury also damages the anti-oxidant defense system, such as SOD, GSH-PX, and CAT. Our Data showed that MDA level was elevated, while and SOD, GSH-PX, and CAT levels were declined in the T2DM group. Consistent with previous findings in a cognitive deficit rat model induced by 3-nitropropionic acid [14], DHM was found to significantly decrease the production of MDA and increase the anti-oxidative activity. Our findings suggest that the ameliorative effects of DHM on DACI may be attributed to the suppression of oxidative stress in T2DM mice.

Furthermore, it has been reported that the expression levels of BDNF were significantly decreased in patients and animals with T2DM [41,42]. The decreased expression of BDNF could exacerbate CI induced by T2DM [10]. BDNF is the most abundant neurotrophin in the brain, which is involved in neuroprotection and neurogenesis crucial for cognition [43–45]. BDNF can specifically bind with Trk-B receptor and subsequently trigger the downstream pro-survival signal pathway [46]. In addition to its neuroprotection effects, BDNF can also regulate glucose metabolism in patients with T2DM [41,47]. In the present research, we observed that the mRNA and protein expression levels of BDNF were decreased in the hippocampus of T2DM mice, whereas DHM treatment significantly up-regulated the expression of BDNF. Additionally, oxidative stress has been reported to negatively correlate with BDNF level [48]. Therefore, we speculate that DHM may exert its ameliorating effects on DACI via enhancing BDNF-mediated neuroprotective signaling, which protects neurons against oxidative damage.

In conclusion, our findings demonstrated that DHM could significantly ameliorate CI and reverse aberrant glucose and lipids metabolism in T2DM mice. Its therapeutic effects could be attributed to the suppression of oxidative stress and enhancement of BDNF-mediated neuroprotection. Our results suggest that DHM is a promising candidate for treating T2DM-induced cognitive dysfunction.

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