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Protective Effect of Buyang Huanwu Decoction on Diabetes-Induced Damage to Hippocampal Neurons by Regulating PI3K-AKT/Bcl-2 Pathway

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ABSTRACT

Diabetic encephalopathy is one of the complications of diabetes, closely related to the degeneration and apoptosis of hippocampal neurons. Buyang Huanwu Decoction (BHD) is a classic traditional Chinese medicine prescription, which has the functions of benefit qi, activing blood circulation and dredging collaterals. To investigate the protective effect and mechanism of BHD on hippocampal neurons in diabetes, a high glucose (HG) induced PC12 cell injury model was established, cell proliferation and apoptosis were detected. The protein expression levels were detected by western blot. Rats with type 2 diabetes mellitus were fed high-fat-sugar diet and low dose of streptozotocin for 4 weeks to observe the body weight, fasting blood glucose, etc. The pathological changes of hippocampal CA1 region were observed through hematoxylin-eosin staining, and the expression of related proteins was detected. The results showed that, after BHD intervention, the degree of apoptosis of PC12 cells injured by HG was significantly reduced, and the cell proliferation was significantly increased. In model group, blood glucose increased significantly, weight loss, oral glucose tolerance was abnormal, while BHD group reversed the above changes. Most nerve cells in BHD group had relatively intact structure and less morphological changes. Immunohistochemistry showed that compared with the model group, the expression of Bcl-2 (B-cell lymphoma 2) in BHD group increased, while the expression of Bax (BCL2-associated X) and caspase-3 decreased. The expression of p-PI3K (phosphorylated phosphatidyl inositol 3- kinase), p-AKT (phosphorylated protein kinase B) and Bcl-2 decreased in model and HG group, while the expression of Bax, Caspase-3, and Cleaved Caspase-3 increased. After BHD intervention, p-PI3K, p-AKT, Bcl-2 expression increased, while Bax, Caspase-3 and Cleaved Caspase-3 expression decreased. In conclusion, BHD may play a protective role on diabetic hippocampal neurons by regulating mitochondria related apoptosis protein and up-regulating PI3K-AKT/Bcl-2 signaling pathway.

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Authors' Contribution
WC designed and implemented the experiment, manuscript writing. WC, YD and LJ analyzed the data. YW and YL provided technical assistance with the experiments. WZ and LZ designed the experiments and made valuable suggestions during the revision of this manuscript.

Key words
Buyang Huanwu decoction, Diabetic encephalopathy, Hippocampal neurons, PI3K-AKT/Bcl-2, Cell apoptosis

INTRODUCTION

Diabetes is a chronic metabolic disease with a large number of patients, leading to a variety of complications (Chen *et al*., 2018; Zheng *et al*., 2018), among which diabetic encephalopathy (DE) is one of the more common complications, which is difficult to be detected in the early stage (Gaspar *et al*., 2016). DE is clinically manifested as memory loss, decreased learning ability, and even dementia (McCrimmon *et al*., 2012). The pathogenesis of DE is complex, mainly involving the degeneration and apoptosis of hippocampal nerve cells, changes of neurotransmitters, damage of synaptic proteins, oxidative stress, inflammatory response, accumulation of glycosylated end products and many other aspects (Biessels and Despa, 2018; Liu *et al*., 2018). The hippocampus is a key region in the central nervous system for memory and learning functions (Squire, 1992; Scoville and Milner, 1957). High glucose (HG) can induce apoptosis and damage of hippocampal neurons, and the decrease of hippocampal neurons can be observed in the rat model of type 2 diabetes (Lang *et al*., 2009). In addition, the high-fat diet of diabetic obese patients also leads to hippocampal neuron damage (Davidson *et al*., 2019). Therefore, hippocampal neuron injury is an important mechanism of DE occurrence and development.

Buyang Huanwu Decoction (BHD) is a commonly

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used traditional Chinese medicine prescription, which has the effects of supplementing Qi, activating blood circulation and dredging collaterals. Studies have shown that BHD can promote the recovery of neurological function in the neuroprotective effect on cerebral ischemia-reperfusion (CIR) injury in rats with cerebral hemorrhage (Shen *et al*., 2020). It can protect hippocampal neurons and improve Alzheimer's disease by participate in synaptic reconstruction, inhibit neuronal damage in hippocampal CA1 region, improve vascular dementia in rats (Li *et al*., 2019; Yang *et al*., 2019), inhibits neuronal apoptosis and improves transient cerebral ischemia by down regulating caspase-3 protein expression in hippocampus (Li *et al*., 2003). *In vivo* and *in vitro* experiments on ischemic stroke showed that BHD promotes neuronal proliferation and differentiation by up-regulating the expression of p-PI3K, p-AKT and p-BAD (Bcl2 antagonist of cell death) proteins in brain tissues (Chen *et al*., 2020). However, the specific mechanism of BHD in preventing hippocampal neuron injury induced by diabetes and treating diabetic encephalopathy remains unclear.

In this study, an *in vitro* model of PC12 cell injury induced by HG was established. Moreover type 2 diabetes mellitus (T2DM) rat model was made high-fat-glucose with low dose of streptozocin (STZ) *in vivo* to observe the protective effect of BHD on HG-injured PC12 cells and the effects on body weight, fasting blood glucose (FBG) and expression levels of apoptosis-related proteins in hippocampus of T2DM rats. This was aimed at investigating the molecular mechanism of BHD's protective effect on DE hippocampal neurons.

MATERIALS AND METHODS

Materials and instruments

BHD was provided by the Pharmacy Department of the Affiliated Hospital to Changchun University of Traditional Chinese Medicine. BHD consists of milkvetch root (120 g), Chinese angelica (6.0 g), peony root (4.5 g), Sichuan lovage rhizome (3.0 g), peach seed (3.0 g), safflower (3.0 g) and earthworm (3.0 g). PI3K (67121-1-lg, 1:2000), AKT (10176-2-AP, 1:2000), p-AKT (66444-1-lg, 1:2000), β-actin (66009-1-lg, 1:5000), Bax (50599-2-lg, 1:5000), Bcl-2 (26593-1-AP, 1:2000), Caspase-3, (66470-2-lg, 1:2000), HRP Goat anti-Mouse IgG (SA00001-1), HRP Goat anti-Rabbit IgG (SA00001-2) was purchased from Axel Biotechnology, Chicago, USA. p-PI3K (CY6427, 1:1000) was purchased from Shanghai Abways Biotechnology Co., Ltd, Shanghai, China. CCK-8 (BA00208) was purchased from Beijing Biosynthesis Biotechnology Co., Ltd, Beijing, China. Fetal bovine serum (FB25015), high glucose Dulbecco's modified Eagle's medium (8121169) was purchased from Gibco, New York, USA. Enhanced BCA Protein Assay KitGlucometer (P0010) was obtained from Shanghai Beyotime Biotechnology Co., Ltd, Shanghai, China.

CCK-8 assay for cell viability

Well-differentiated PC12 cells were provided by the School of Basic Medicine of Jilin University, and were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. BHD were decocted with 300 mL water at 100°C for 1 h, repeated 3 times to obtain the aqueous extract, then were combined and centrifuged. The supernatants were dried under vacuum to produce a brownish powder. For biological studies, 10 mg/mL stock concentration decoction of BHD was filtered (0.2 μm), sterilized, and diluted. PC12 cells at logarithmic growth stage were inoculated into 96-well plates (1.5×10^3 per well), cultured for 12-24 h, and divided into 4 groups. Except for the control group, the culture medium of all other groups was discarded. Different drugs with 100 mmol/L HG culture medium (HG), 800 μg/mL BHD, 400 μg/mL BHD and 2.0 mmol/L metformin (metformin positive control group, Met) were added respectively and set as HG, BHD-H, BHD-L, Met groups. After 48 h of culture, the absorbance was measured at 450 nm using a microplate reader.

Flow cytometry assay for apoptosis

PC12 cells at logarithmic growth stage were inoculated evenly in 6-well plates, 1×10^5 cells per well. After 12-24 h of culture, treated with BHD and Met for 48 h use simultaneously stimulation with HG. Then, PC12 cells were harvested and washed twice with cold PBS (phosphate buffer saline). Subsequently, Annexin V-FITC/PI kit instructions were followed, the treated PC12 cells were suspended with 300 μL 1×binding buffer, annexin V-FITC 5 μL and PI 10 μL, and thoroughly mixed. The reaction was conducted at room temperature for 10min under dark conditions. Flow cytometry (easyCyte, Guava, USA) was used for detection within 1 h.

Animals and treatment

Sixty specific-pathogen free (SPF) male SD rats (100±20g) were provided by Liaoning Changsheng Biotechnology Co., LTD. All rats were fed at 22±2°C, relative humidity 45-65%, light/dark cycle 12 h/12 h, with free food and water intake, and were adaptively fed for 3 d. Ten rats were randomly selected as the control group (Con), the others were fed with high-fat-sugar diet for 4 weeks before modeling.

Before modeling, rats were fasted without water for 12 h, and 1% streptozocin (STZ) solution was intraperitoneally injected at 35 mg/kg in citrate buffer at the lower left side of control group. After 30 min of injection, the rats were normally fed with water. After 72 h, FBG content was measured. If FBG ≥16.7 mmol/L, the T2DM model was considered as successful. The rats were further randomly divided into 5 groups: T2DM model group (DM), BHD high dose group, middle dose group, low dose group and metformin group: 10 rats in each group. Control group and DM group were given normal saline gavage. BHD-H, BHD-M and BHD-L groups were given 59.81, 29.90, 14.95 g/kg, and metformin group (met) was given 89.25 mg/kg. After continuous intervention for 8 weeks, the state of the rats were observed at ordinary times. FBG and body weight were tested regularly every week.

Oral glucose tolerance test (OGTT)

After 8 weeks of administration, the rats were fasted for 12 h and given 40% glucose solution at the volume of 5 mL/kg by intragastric administration. Blood glucose was measured at 0, 0.5, 1.0, 1.5 and 2.0 h, respectively; and the OGTT curve was prepared accordingly.

Hematoxylin-eosin (H & E) staining

After gavage for 8 weeks, rats were anesthetized with pentobarbital sodium and fixed in supine position. Under anesthesia, the brain was cut off, rinsed with normal saline and dried with filter paper. Part of the hippocampus was preserved in liquid nitrogen, and the rest was fixed in 4% paraformaldehyde, conventional paraffin sections were prepared and embedded for subsequent experiments.

Sections were routinely dewaxed, hydrated, nucleated with hematoxylin, differentiated with 10% hydrochloric acid alcohol, reverse-blue with ammonia water for several seconds, washed with distilled water, stained with eosin for 3 min, dehydrated with ethanol of various gradients, transparent with xylene, sealed with neutral gum, and observation under a microscope.

Immunohistochemistry

Paraffin sections were dewaxed, gradient ethanol hydration, washed with distilled water, immunohistochemistry pen was used to delineate the drop range, endogenous peroxide blocker was added to the delineated area, incubated for 10 min at room temperature. Then, non-specific stain blocker was dropped and incubated for 10 min at room temperature, then washed with PBS. Primary antibody (1:400) was added, incubated at 37°C for 2 h, washed with PBS, then biotin-labeled sheep anti-rabbit/mouse IgG polymer was added, incubated at room temperature for 10 min. Washed with PBS, streptomycin anti-biotin-peroxidase was added, and incubated at room temperature for 10 min. Washed with PBS, DAB was added, and incubated at room temperature for 3-5 min. Rinsed with water, redyed for 2 min and the tablet was sealed. Five sections in the whole hippocampus were selected for analysis. The positive cells of related apoptotic proteins in the CA1 region were counted and averaged.

Western blot analysis

PC12 cells cultured to logarithmic stage and hippocampal tissue from T2DM rats were collected and lysed in radioimmunoprecipitation (RIPA) assay buffer. Then centrifuged at 12,000 rpm for 10 min, and the supernatants were collected for total protein concentration analysis by BCA assay. 20 μL protein sample was subjected to SDS-PAGE electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membrane. After being blocked with 10% skim milk for 1 h, the membranes were incubated with primary antibodies at 4°C overnight. After washing with Tris-buffered saline containing 0.1% tween-20 (TBST), the membranes were incubated with secondary antibody (Horse radish Peroxidase, HRP) for 1 h at room temperature. After an enhanced chemiluminescence (ECL) reaction for 15 s, the protein bands were visualized using ChemiDoc XRS Imaging System (Bio-Rad, Hercules, CA, USA). With β-actin as an internal reference, the gray value was calculated by ImageJ 1.8.0 software (NIH, Bethesda, MD, USA).

Statistical analysis

SPSS 22.0 software (International Business Machines Corporation, New York, USA) was used for statistical analysis. $P<0.05$ showed that the difference was significant.

RESULTS

BHD treatment attenuated HG-induced cell injury in PC12 cells

The proliferation of model group was significantly decreased ($P<0.01$) while the cell proliferation of BHD group was increased significantly ($P<0.01$) (Fig. 1A-B). PC12 cells in model group showed early apoptosis ($P<0.01$). The apoptosis of BHD groups was decreased significantly ($P<0.01$) (Fig. 1C). These results suggest that BHD can promote PC12 cell proliferation and inhibit HG induced apoptosis in PC12 cells.

Effects of BHD treatment on STZ-induced body weight and blood glucose in T2DM rats

After 8 weeks of intervention, body weight in model

group was decreased significantly (P<0.01) (Fig. 2A) while FBG was increased significantly (P<0.01) (Fig. 2B). Body weight in three doses of BHD groups were increased significantly (P<0.01) (Fig. 2A) and FBG was decreased significantly (P<0.01) (Fig. 2B). In OGTT tests, the FBG levels were significantly increased at 0, 0.5, 1.0, and 1.5 h (P<0.01) (Fig. 2C); The FBG levels in the BHD dose groups were significantly decreased at 30 min, 60 min and 90 min (P<0.01) (Fig. 2C). The results indicate that BHD has a relieving and therapeutic effect on diabetes.

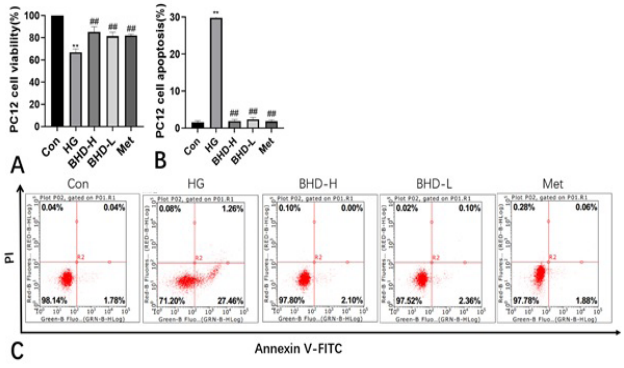

Fig. 1. Effect of BHD on PC12 cell viability, (A) and PC12 cell apoptosis (B). Apoptosis was detected by flow cytometry (C). Results are shown as the mean ± SD (n = 3); ** P<0.01, vs. the control group; ## P<0.01, vs. the HG group. Con, control group; HG, 100 mmol/L high glucose group; BHD-H, 800 μg/mL BHD group; BHD-L, 400 μg/mL BHD group; Met, 2.0 mmol/L metformin group.

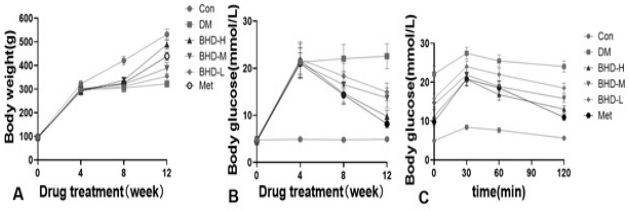

Fig. 2. Effect of BHD on body weight (A), fasting blood glucose (B) and analysis of OGTT test in 8 weeks (C). Con, control group; DM, T2DM model group; BHD-H, 59.81 g/kg BHD group; BHD-M, 29.90 g/kg BHD group; BHD-L, 14.95 g/kg BHD group; Met, 89.25 mg/kg metformin group.

Effect of BHD on hippocampal neurons of T2DM rats

The hippocampal CA1 neurons had normal morphology in control group. The cytoplasm is abundant, and the color is uniform. There is no nuclear shrinkage or dissolution. Compared to control group, the cells in the hippocampal CA1 area of model group were significantly reduced, disordered, and the nuclei were hyperchromatic, the neuronal space was widened. Most of the nerve cells in BHD groups and metformin groups were relatively intact, with light changes in cell morphology and less nuclear shrinkage. The improvement degree of BHD on hippocampal CA1 area was gradually enhanced with the increase of dose (Fig. 3A).

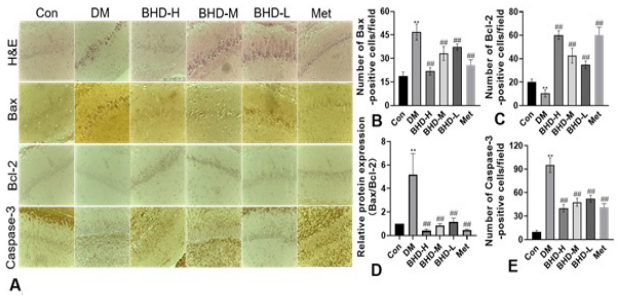

Fig. 3. Effect of BHD on representative microscopical images of cerebral hippocampus (A), showing CA1 region from each group, (B) number of Bax, Bcl-2 (C), Bax/Bcl-2 (D), caspase-3 positive cells/field (E). Tissue sections of cerebral hippocampus CA1 region were stained with H & E and IHC (×200 magnification). ** P <0.01, vs. the control group; ## P<0.01, vs. the DM group.

Immunohistochemistry showed positive expression of Bax, Bcl-2 and caspase-3 proteins a brown, brownish yellow or light yellow. The staining of Bax, Bcl-2 and caspase-3 positive neurons in hippocampal neurons of normal group was uniform and the contours were regular. The number of Bax positive neurons in the hippocampus of model group was significantly increased (P<0.01) (Fig. 3B), with darker staining and less clear contour. The number of Bcl-2 positive neurons decreased significantly (P<0.01) (Fig. 3C) and the staining was shallow. The positive expressions of Bax and caspase-3 in BHD administration groups were significantly decreased (P<0.01) (Fig. 3B, D) and the positive expression of Bcl-2 was significantly increased (P<0.01) (Fig. 3C, E). In this study, most nerve cells in each BHD administration group were relatively intact in structure, with light changes in cell morphology. The improvement degree of BHD on hippocampal CA1 area was gradually enhanced with the increase of dose.

BHD treatment altered the HG-inhibited PI3K-AKT/Bcl-2 pathway in PC12 cells and T2DM rats

In vitro, HG reduced PI3K and p-AKT, but had no significant effect on AKT (P<0.01) (Fig. 4A-E). The expression quantities of Bax, caspase-3 and cleaved caspase-3 were significantly increased (P<0.01) (Fig. 4A, F, J). The protein expressions of PI3K, p-AKT and Bcl-2 in BHD dose groups were significantly increased (P<0.01)

(Fig. 4A, B, E, G), while the protein expressions of Bax and Caspase-3 were significantly decreased (P<0.01) (Fig. 4A, F, J).

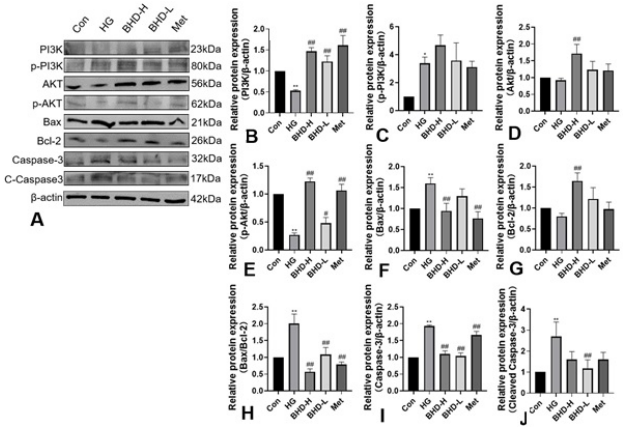

Fig. 4. (A) BHD treatment inhibited HG-induced apoptosis pathway in PC12 cells. The density of PI3K, p-PI3K, AKT, p-AKT, Bax, Bcl-2, and Caspase-3 were measured by Western Blot. (B) Quantitative data of PI3K, (C) p-PI3K, (D) AKT, (E) p-AKT, (F) Bax, (G) Bcl-2, (H) Bax/Bcl-2, (I) Caspase-3, and (J) Cleaved Caspase-3 are presented. Data are shown as mean ± SD (n=3). * P <0.05, ** P <0.01, vs. the control group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, vs. the HG group.

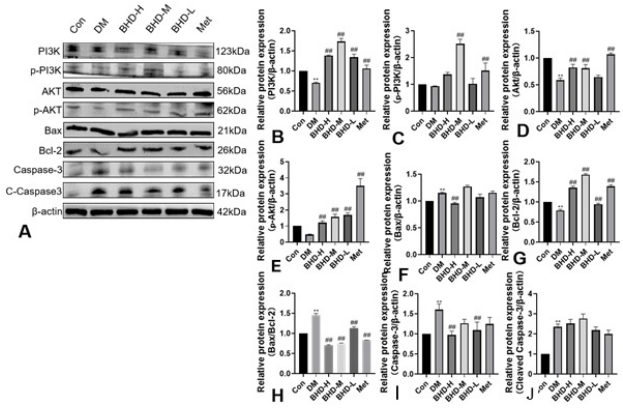

Fig. 5. (A) BHD treatment inhibited STZ-induced apoptosis pathway in rats with DM. The density of PI3K, p-PI3K, AKT, p-AKT, Bax, Bcl-2, and Caspase-3 were measured by Western Blot. (B) Quantitative data of PI3K, (C) p-PI3K, (D) AKT, (E) p-AKT, (F) Bax, (G) Bcl-2, (H) Bax/Bcl-2, (I) Caspase-3, and (J) Cleaved Caspase-3 are presented. Data are shown as mean ± SD (n=3). ** P <0.01, vs. the control group; $^{\#\#}P$ < 0.01, vs. the DM group.

In vivo, the expression quantities of PI3K, AKT, and Bcl-2 in the model group were significantly decreased (P<0.01) (Fig. 5A-E), the expression quantities of Bax, Caspase-3, and Cleaved Caspase-3 were significantly increased (P<0.01) (Fig. 5A, F-J). The protein expressions of PI3K, p-PI3K, AKT, p-AKT and Bcl-2 in BHD dose groups were significantly increased (P<0.01) while the protein expressions of Bax and Caspase-3 were significantly decreased (P<0.01). These results suggest that BHD may improve hippocampal neuron injury by regulating PI3K-AKT/Bcl-2 pathway.

DISCUSSION

Until 2019, there were about 463 million diabetes patients in the world, with an average of 1 in 11 people suffering from diabetes, and about 90% of them belong to type 2 diabetes (Saeedi *et al*., 2019). HG can induce the decrease of related protein Bcl-2/Bax ratio in hippocampus, leading to neuronal apoptosis. PC12 cells are commonly used to study DE and brain neurodegenerative diseases (Wang *et al*., 2021). In this experiment, HG inhibited the proliferation and led to PC12 cells apoptosis. After BHD intervention, the survival rate of cells was significantly increased and the degree of apoptosis was significantly reduced. Metformin is a commonly used clinical hypoglycemic drug, which can reduce the weight of obese mice fed with high fat and high sugar (Coll *et al*., 2020). It can protect neurons in hippocampal CA1 region of rats, promote neuronal differentiation and regeneration, regulate PI3K-AKT signaling pathway to inhibit neuronal apoptosis and play a neuroprotective role (Wang *et al*., 2020). Therefore, in this study, metformin group is positive control. *In vivo* was prepard T2DM rat model, using high-fat-sugar feeding with low-dose STZ. After modeling, the blood glucose of the rats increased significantly, the weight of the model group decreased, and the oral glucose tolerance test was abnormal. Diabetes can lead to progressive damage of brain and neurons (Kong *et al*., 2018; Toth, 2014), and pathophysiological changes will occur in the hippocampal area of diabetic rats induced by STZ (Sun *et al*., 2021). The neurons in the hippocampal CA1 area of the model group were significantly reduced and disordered. Most of the nerve cells in BHD treatment groups were relatively intact and their morphological changes were light, indicating that BHD has a protective effect on neurons in hippocampal CA1 region.

Mitochondria are the central regulator of neurons, and their damage is a key factor in the occurrence of neurodegenerative diseases (Khacho *et al*., 2019). Bax, Bcl-2 and Caspase-3 are all mitochondria related apoptosis proteins, which mainly exist in the cytoplasm of non-apoptotic cells and translocate to mitochondria after apoptotic signal stimulation (Santos *et al*., 2019). Induced by high glucose, the expression of apoptosis proteins in

cells were changed and the expression of upstream p-AKT decreased in PC12 cells (Yan *et al*., 2020). In this study, protein expression in HG group PC12 cells was consistent with previous studies, while protein expression in treatment group was contrary to the above. These results show that BHD may play a neuroprotective role by regulating Bcl-2 pathway related proteins. PI3K-AKT is the upstream signaling pathway of Bcl-2 pathway and an important pathway leading to the occurrence and development of DE (Lei *et al*., 2021). It can regulate neuronal apoptosis (Nan *et al*., 2019), inflammation, oxidative stress and other mechanisms, and inhibit neuronal damage in hippocampus (Xing *et al*., 2020). Studies have shown that of apoptosis of hippocampal neurons in aging model rats is inhibited and regulate the expression of apoptosis-related proteins (Tian *et al*., 2019). Decreased PI3K/AKT pathway activity can lead to significantly decreased p-Akt and Bcl-2 protein expression, thereby increasing hippocampal neuron apoptosis (Wang *et al*., 2019). In this study, PI3K-AKT /Bcl-2 pathway was detected in PC12 cell and T2DM rat. In the model group and HG group, p-PI3K related protein expression was decreased, and Bax related protein expression was increased, which was consistent with previous results. After BHD intervention, p-PI3K related protein expression increased, Bax related protein expression decreased. These results indicate that BHD may play a protective role on diabetic hippocampal neurons by regulating mitochondrial related apoptosis proteins and regulating PI3K-AKT /Bcl-2 signaling pathway.

CONCLUSION

In this study, BHD has protective effect on PC12 cells *in vitro*. *In vivo*, BHD can increase body weight, decrease blood glucose, improve hippocampal neurons in T2DM rats. The results suggest that BHD can protect hippocampal injury and regulate and treat diabetes in T2DM rats. It may be realized by regulating the expression of mitochondrial apoptosis-related proteins in PI3K-AKT/ Bcl-2 signal pathway.

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Ethical compliance

This paper was certified by the animal care and welfare committee of Changchun University of Chinese Medicine (20190141).

Statement of conflict of interest

The authors have declared no conflict of interest.

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