Nao-Fu-Cong ameliorates diabetic cognitive dysfunction by inhibition of JNK/CHOP/Bcl2-mediated apoptosis in vivo and in vitro

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[ABSTRACT] Chinese herbal compound Nao-Fu-Cong (NFC) has been mainly used to treat cognitive disorders in Traditional Chinese Medicine (TCM). The present study aimed to investigate whether its neuroprotective effects might be related to the inhibition of JNK/CHOP/Bcl2-mediated apoptosis pathway or not. We randomly assigned STZ (60 mg·kg⁻¹)-induced diabetic rats into control group, diabetic model group and NFC groups (low-dose, medium-dose and high-dose). The primary culture of hippocampal neurons were transferred into different culture media on the third day. The cells were then divided into control group, high-glucose group, NFC (low-dose, medium-dose and high-dose) groups, CHOP si-RNA intervention group, JNK pathway inhibitor SP600125 group and oxidative stress inhibitor N-acetylcysteine (NAC) group. NFC significantly improved the cognitive function of diabetic rats, and had neuroprotective effect on hippocampal neurons cultured in high glucose. Further research results showed that NFC could reduce the apoptosis of hippocampal neurons in rats with diabetic cognitive dysfunction. NFC had inhibitory effects on CHOP/JNK apoptosis pathway induced by high glucose, and also decreased the levels of ROS and increased the mitochondrial membrane potential. These suggested that the neuroprotective effect of NFC might be related to the inhibition of CHOP and JNK apoptotic signaling pathways, and the cross pathway between oxidative stress and mitochondrial damage pathway.

[KEY WORDS] Nao-Fu-Cong; Apoptosis; High glucose; Hippocampal neurons


Introduction

Diabetes mellitus (DM) is one of the most common diseases. About 347 million people have diabetes worldwide, and the number is increasing [¹]. It has been established that DM is associated with cognitive dysfunction. Mijnhout, et al. [²] proposed a new term “diabetes-associated cognitive decline “ (DACD). Considerable attention has been given to DACD in the last decade [¹]. The mechanisms underlying the development of DACD have not been completely elucidated. Previous studies have demonstrated that cerebrovascular changes, disturbances of neuronal Ca²⁺ homeostasis, oxidative stress and impaired cerebral insulin signaling are the underlying causes of DACD [⁴⁴]. It also has been confirmed that the degeneration and apoptosis of hippocampal neurons played an important role in the learning and memory deficits in diabetic animals [⁷-⁹].

To date, apoptosis has been recognized not only in the pathogenesis of diabetes, but also in multiple neurodegenerative diseases and cerebral ischemia reperfusion injury [¹⁰, ¹¹]. C/EBP homology protein (CHOP) pathway is a key apoptotic pathway. Recent studies show that CHOP pathways can aggravate neuronal apoptosis by interacting with oxidative stress and mitochondrial damage pathways [¹²-¹³]. Moreover, CHOP-dependent apoptosis-mediated cell death is implicated in several neurodegenerative diseases [¹⁴, ¹⁵] and promote the diabetic cognitive impairment [¹⁶]. Studies showed that CHOP induces neuronal apoptosis through multiple pathways, including JNK, GSK3/β and Caspase-12 pathways, and through interaction with mitochondrial apoptotic pathways. CHOP pathway is widely confirmed as a key player in various neuronal injury models. Upon activation, this protein will then lead to neuronal apoptosis by down-regulating Bcl2 expression, promoting transcription of death receptor 5 (DR5), inducing TRB3 expression, and upregulating Puma expression, among others.

Nao-Fu-Cong (NFC) decoction has been mainly used to...
treat cognitive disorders in TCM. It is a safe and useful prescription. Our previous studies showed that NFC could improve learning and memory abilities of diabetes rats. It also exhibited protective effect on cognition deficits of cerebral ischemic mice, which might be related to enhancement of the cerebral super oxygen dehydrogenises (SOD) and glutathione peroxidase (GSH-Px) activities, inhibition of the activity of N-methyl-D-aspartic acid (NMDA) receptor and increasing cholinergic system activity [17]. Therefore, our study aimed to investigate whether NFC could exert its neuroprotective effects by CHOP and JNK apoptosis pathways and their interaction with oxidative stress and mitochondrial damage pathways.

Materials and Methods

Materials

Newborn rats [the Peking University Health Science Center (Beijing, China)]; TUNEL Apoptosis Assay Kit (06432344001, Roche, USA); Annexin v/PI Kit (11988549001, Sigma, USA); N-acetylcysteine (NAC) was purchased from TCI chemicals (616-91-1, Shanghai, China); CHOP si-RNA (Invitrogen, CA, USA); JNK Pathway Inhibitor SP600125 (molecular formula of C14H8N2O, molecular weight of 220.32, purity ≥ 98%, Art.No. S5567, Sigma, USA); DCFH-DA fluorescence probe (4091-99-0, Sigma, USA); Mitochondrial Membrane Potential Assay Kit (C2006, Beyotime, China); Hoechst fluorescent dyes (33342, Sigma, USA); Rabbit anti-rat p-JNK, JNK, CHOP, Bcl-2, Caspase-3, Caspase-9 multiclonal antibodies (Santa Cruz, USA); All cell culture reagents were from Peking union cell center (Beijing, China). Y maze and Morris water maze were provided by Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. The NFC consists of Radix Ginseng, Radix Salviae Miltiorrhizae, Radix Polygogni multiflori, Hirudo, Rhizoma acori tatarinowii, Coptis chinensis and Portia cocos. These herbs were processed into granules by Beijing Kangrentang Pharmaceutical Co., China and prepared into aqueous solution.

In vivo studies

Animal model induction

50 Male rats of specific pathogen free (SPF) grade weighed 180–200 g were provided by Experimental Animal Center of PLA Military Academy of Medical Sciences (SCXK 2016-0006, Beijing, China). The rats were randomly divided into five groups of 10 rats each: low-dose NFC group, medium-dose NFC group, high-dose NFC group, diabetic model group and control group. The rats in NFC intervention groups and diabetic model group were intraperitonially injected 60 mg kg⁻¹ STZ, and those in control group were injected the same volume of buffer solution. After the 1st week, blood was collected from their tail vein and blood glucose was measured with potable glucometer. A blood glucose concentration ≥ 16.7 mmol L⁻¹ indicated a successful model. The rats in low-dose NFC group (0.724 g kg⁻¹ d⁻¹), medium-dose NFC group (1.448 g kg⁻¹ d⁻¹) and high-dose NFC group (2.896 g kg⁻¹ d⁻¹) were administered the medication for 12 weeks, respectively. The rats in control group and diabetic model group were administered same volume of distilled water.

Preparation of NFC-containing serum

The SD rats were intragastrically administered with NFC twice daily for 3 days (4.667 g kg⁻¹ per day). Two hours after the last administration, arterial blood was collected from the internal carotid arteries and sat at room temperature for 2 h. The blood was then centrifuged at a speed of 4000 r min⁻¹ for 10 min to separate the drug-containing serum. The drug-containing serum was filtered through 0.22 μm filters, inactivated at 56 °C for 30 min, and stored at −20 °C before use.

High-pressure liquid chromatography (HPLC) analysis of NFC

High performance liquid chromatography was used for analyzing the quality of formula by monitoring the four active components in it. The standards used for the quantitative analysis of QHD were chlorogenic acid, polygonin, resveratrol, and jasminoidin, which are currently recommended for analyzing QHD. These standards were obtained from the Chinese National Centre for Quality Control of Traditional Chinese Medicine, China. The QHD and standard compounds were dissolved in methanol, filtered through nylon filters, and analyzed in HPLC. Liquid chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies) equipped with a quaternary solvent delivery system, online degasser, autosampler, and column temperature controller. A mixture of methanol and water as mobile phase in a gradient mode was used in Agilent ZORBAX Eclipse XDB-C18 (4.6 mm × 250 mm, 5 μm) column. The gas flow was 1.8 L min⁻¹ with a 1 mL min⁻¹ flow rate and the column was maintained at 40 °C. The detection was done at 254 nm wavelength.

Morris water maze test

The maze consisted of a black circular pool made of stainless steel (150 cm in diameter) and was filled with water with a temperature of 20 ± 1 °C. A platform was put into the pool and submerged 2 cm below the water surface. Black ink was then poured into the water to create a contrast with the white rats. The platform was placed in the middle of one quadrant and the rats entered the water lying on their back from the diagonally opposite quadrant1/2. Each training session lasted for 120 s. A monitoring system above the pool was connected to the computer that kept track of the rats and calculated the time they spent reaching the platform (latency). After completion of the task, it automatically stopped recording and the rats were allowed to rest for 20 s. If the rats failed to located the platform within 120 s, the monitor stopped and they were also placed on the platform for 20 s. The rats were trained for three days and each received learning trials on the fourth and fifth days for 120 s.

Y maze

Y maze took shape of a ‘Y’ and was made of PVC boards covered with black films. The three arms were named...
These steps were repeated for 40 cycles. Steps to generate of s, annealing at 60 °C for 20 s, extension at 72 °C for 30 s.

GAGGCAGGGATGAT-3′; PCR amplification curve: initial GTATCGTGGAAGGACTCA-3′; Anti-sense: 5′-CCAGAT-TGC-3′; Fragment length: 195 bp; GAPDH Sense: 5′-TG-GACTGAGG-3′; Anti-sense: 5′-CTGCTCCTTCTCCTTCA3′; Anti-sense: 5′-CGGTAGCGACGAGAGAAGTC-3′; Genebank: Bcl2 Sense: 5′-CTGGCATCTTCTCCTTCCAG-3) according to the mRNA sequences provided in NCBI kit. The primers were designed as followed (software Primer tained following the instructions of the reverse-transcription kit. The concentration and purity was measured. cDNA was ob-

Real-time-PCR

At the 12th week, the rats were anesthetized with intra-

Western blot analysis

In vitro studies

Isolation, purification and incubation of hippocampal neurons

All experimental procedures were carried out in accordance with international guidelines on the ethical treatment of animals, as well as with the experimental standards of the Chinese Academy of Medical Sciences. Animals were sacrificed by cervical dislocation, and all efforts were made to minimize the number of animals used and their suffering. Hippocampi were dissected from the brain on ice and minced in sterile ice-cold D-Hanks’ with the blood vessels and meninges carefully removed. The tissues were digested with 0.25% trypsin for 15 min at 37 °C and then the digestion procedure was stopped by adding 5 mL fetal bovine serum (FBS). Cell suspension was screened with a 200 mesh cell strainer and separated by density gradient centrifugation at 702.4 g for 20 min. Then, the cell suspension containing the desired cell fractions, was centrifuged in D-Hanks’ for 5 min at 395.1 g and resuspended in 15–20 mL DMEM medium. After determining the cell density using a hemacytometer, the cells were then plated on poly-D-lysine (Sigma, USA) coated-glass coverslips at a density of 5 × 10⁵ cell/mL and maintained at 37 °C in a humidified 5% CO₂ incubator (Forma, USA). Neurons were cultured in DMEM medium and supplemented with 10% FBS, 20 mmol·L⁻¹ sodium pyruvate, 1 mmol·L⁻¹ glutamine, 100 U·mL⁻¹ penicillin, 100 μg·mL⁻¹ streptomycin, 10 ng·mL⁻¹ NGF (Millipore, USA). After 3 days, the cells were divided into healthy control group, high-glucose group, low/medium/high-dose NFC group, CHOP si-RNA intervention group, JNK pathway inhibitor SP600125 (10 μmol·L⁻¹) group and oxidative stress inhibitor N-acetylcysteine group (NAC, 10 mmol·L⁻¹). The cells in control group were cultured on complete medium and others were cultured on medium with a final glucose concentration of 50 mmol·L⁻¹.

TUNEL assay

We performed in situ labeling of TUNEL-positive nuclei using the In Situ Cell Death Detection Kit, POD (Roche, USA) following the manufacturer’s protocol. In brief, (1) the cells were rinsed with 0.01 mmol·L⁻¹ PBS and incubated with 4% paraformaldehyde for 1 h at room temperature for fixation; (2) the cells were washed with 0.01 mol·L⁻¹ PBS three times for 2 min each, and incubated with 0.1% TritonX-100 for 3 min at 4 °C; (3) the cells were washed with 0.01 mol·L⁻¹ PBS three times for 2 min each. 20 μL DNase I was added to the positive control group, and the cells were incubated for 10 min at room temperature; (4) 20 μL TUNEL reaction solution was added to all the samples and the cells were placed in the humidifying box in the dark and incubated for 60 min at 37 °C; labeling solution was added to the negative control group; (5) the cells were rinsed with 0.01 mol·L⁻¹ PBS three times...
times for 2 min each. 20 μL POD converter was added to each cover glass and the cells were placed in the humidifying box and incubated for 30 min at 37 °C. The cells were then rinsed with 0.01 mol·L⁻¹ PBS three times for 2 min each, and their nuclei were visualized with 20 μL 0.01 mol·L⁻¹ DAB solution on light microscope. Once specific brownish-yellow particles were identified, the cells were rinsed with distilled water to terminate the reaction. The slides were then re-stained with hematoxylin for 10 s, prepared with water soluble mounting medium and viewed with light microscope.

Cells with specific brownish-yellow particles were TUNEL positive, i.e. apoptotic cells. Pictures were collected with Leica microscope and imaging collecting system, and were analyzed using Image-Pro Plus 6.0 software. The process was repeated three times, among each of which 5 fields were randomly chosen to count the number of positive cells and total cells, and to calculate the percentage of apoptotic cells.

Annexin V/PI/Hoechst triple staining

Annexin V-FITC/PI staining was performed with a kit from Sigma-Aldrich (St. Louis, MO, USA) according to the manufacturer’s protocol. Briefly, cells were harvested and resuspended in binding buffer (106 cells/mL) after being stained with Hoechst 33342 (Sigma, USA).

After the addition of appropriate amount of binding buffer (1 : 9), Annexin V (1 : 100) and PI (1 : 50) into each well, the kit was placed in the dark at room temperature for 10 min. It was then observed and photographed with laser scanning confocal microscope. The pictures were analyzed with Volocity Demo software. The process was repeated three times, among each of which 5 fields were randomly chosen to count the number of apoptotic cells and total cells and to calculate the percentage of apoptotic cells. Apoptotic cells are Annexin V stain-positive and PI stain-negative.

Intracellular ROS measurement

The fluorescent probe DCFH-DA, which can be oxidized to the highly fluorescent compound dichlorofluorescein (DCF) was employed to measure intracellular reactive oxygen species. Briefly, after incubation, cells were incubated with 10 μmol·L⁻¹ DCFH-DA at 37 °C for 30 min and then were washed twice with ice-cold PBS for 5 min. Cellular fluorescence intensity was detected using a live cell imaging system (Olympus LCS SYSTEM, Japan) with excitation at 488 nm and emission at 527 nm. The experiments were repeated three times independently.

Mitochondrial membrane potential (Δψm) measurement

The mitochondrial membrane potential (Δψm) of hippocampus neurons was monitored using the fluorescent, lipophilic and cationic probe JC-1 (Beyotime, China). Briefly, cells were cultured in 8-well plates (Thermo Fisher Scientific, USA) and incubated with JC-1 staining solution (5 μg·mL⁻¹) at 37 °C for 20 min.

After incubation, the supernatant was discarded and the samples were washed twice with JC-1 staining buffer. 0.2 mL of culture solution was added. All samples were observed and photographed on the live cell station with laser confocal microscope. The intensity of green fluorescence emitted by the JC-1 monomer was detected by the first channel. The excitation wavelength was 488 nm and the emission wavelength was 525 nm. The intensity of red fluorescence emitted by the JC-1 polymer was detected by the second channel. The excitation wavelength was 488 nm and the emission wavelength was 590 nm. The ratio of red/green fluorescent intensity was measured with the Volocity Demo software. The process was repeated three times. For each group of samples, 5 microscopic fields were selected randomly. The ratio of red/green fluorescent intensities represented the mitochondrial membrane potential of the hippocampal neurons.

Statistical analysis

All data were analysed with SPSS 17.0 software. Prior to analysis, One Sample Kolmogrov-Smirnov Z test was used to examine whether the data followed normal distribution. Normally distributed data were presented as mean ± SEM. One-Way ANOVA were used to compare between two or more independent groups. K Independent Samples Test were used for non-normal data. Statistical significance was considered at P < 0.05.

Results

Effects of NFC on the capacity for learning and memory of the diabetic rats

First, the Morris water maze test was used to explore the cognitive function of STZ-induced diabetic rats. Upon completion of training, trials were performed on the 4th and 5th day. As shown in Fig. 1A, the latency of the rats was signific-

![Fig. 1](image-url)

Fig. 1 Effects of NFC on the learning and memory capacity of the diabetic rats. Morris water maze (A) and Y maze (B) are shown. Data are expressed as means ± SEM (n = 10 per group), *P < 0.05.
and dendrites shrunk and the nuclei shifted to the side. The yellow particles in the high-glucose group. Their cell bodies number of neurons contained nuclei with specific brownish-

Effects of NFC on apoptosis of hippocampal neurons in high glucose

Then, the role NFC played in the apoptosis of hippocampal neurons in the experimental diabetic rats was tested. Apoptotic rate of hippocampal neurons of diabetic rats and the effects of NFC could be observed with TUNEL assay. Presence of specific brownish-yellow granules indicated TUNEL positive cells, i.e. apoptotic cells. Compared with those in the control group, more nuclei of the cells in the diabetic model group contained specific brownish-yellow granules. The number of cells with specific brownish-yellow granules was smaller in medium-dose and high-dose NFC groups than that in the diabetic model group (Fig. 2A).

As illustrated in Fig. 2B, the expression of CHOP, p-JNK, Caspase-3 and Caspase-9 protein was higher (P < 0.01) in the diabetic model group than that in the control group, and the expression of Bcl2 protein in the diabetic model group was significantly lower (P < 0.01). Compared with that in the diabetic model group, the expression of CHOP, p-JNK, Caspase-3 and Caspase-9 protein was significantly decreased in NFC-H, NFC-M and NFC-L groups (P < 0.01). The NFC-H and NFC-M groups significantly showed an upregulated level of Bcl2 protein expression compared with the diabetic model group (P < 0.01).

Real-time PCR results revealed that the expression of CHOP and JNK gene was higher (P < 0.01) and the expression of Bcl2 gene was lower (P < 0.01) in the diabetic model group than that in the control group. Compared with that in the diabetic model group, the expression of CHOP and JNK gene decreased significantly in the NFC-H, NFC-M and NFC-L groups (P < 0.01). The NFC-H group significantly showed an upregulated level of Bcl2 gene expression compared with the model group (P < 0.01).

Effects of NFC on apoptosis of hippocampal neurons in high glucose

We observed the effect of NFC-containing serum on high glucose-induced apoptosis in cultured hippocampal neurons using the TUNEL staining. DAB stain revealed a number of neurons contained nuclei with specific brownish-yellow particles in the high-glucose group. Their cell bodies and dendrites shrunken and the nuclei shifted to the side. The cells in healthy control group and drug intervention group had robust bodies. Their dendrites were extended only a few nuclei had specific brownish-yellow stain. Based on statistical analysis, the apoptotic rate in the high-glucose group was significantly higher (54.2 ± 12.6%) than that in the control group (9.6 ± 3.1%). And the rate was lower in NFC-H group (13.6 ± 1.9%), CHOP siRNA intervention group (12.8 ± 1.1%), JNK group (14.6 ± 1.9%) and NAC intervention group (17.1 ± 1.71%, Fig. 3A).

Annexin V/PI/Hoechst triple staining: The proportion of Annexin V (+)/PI (−) neurons (i.e. apoptotic neurons) was higher in the high-glucose group, and the blebbing phenomenon was more apparent. The proportion of Annexin V (+)/PI (−) neurons in the control group (12.97% ± 1.35%) and drug intervention group was lower. The results of statistical analysis was in line with those in TUNEL staining. The apoptotic rate in the high-glucose group increased significantly (49.3% ± 2.39%) compared with that in the control group (P < 0.01). And the rate was also lower in NFC, CHOP siRNA, JNK and NAC intervention group (P < 0.01). There was no significant difference between drug intervention groups (P > 0.05, Fig. 3B).

The results of Western Blot were exhibited below. Compared with that in the control group, the level of phosphorylated JNK and CHOP protein was higher (P < 0.05) whereas that of Bcl-2 protein was lower (P < 0.05) in the high-glucose group. CHOP expression in all drug intervention groups was significantly lower (P < 0.05), and the expression of Bcl-2 higher (P < 0.05). The level of phosphorylated JNK decreased significantly in NFC-H, NFC-M, CHOP si-RNA intervention, JNK inhibitor and NAC groups. Regulation of the expression of phosphorylated JNK, CHOP and Bcl-2 protein was better in NFC-H group than that in the CHOP si-RNA intervention, JNK inhibitor and NAC group (P < 0.05, Figs. 3C and 3D).

The results of real-time PCR were largely in line with those of Western Blot. The expression of CHOP mRNA was higher in the high-glucose group than that in the control group (P < 0.05). The neuroprotective effects were superior in the high-dose NFC group than those in the NAC and JNK inhibitor groups (P < 0.05), and the neuroprotective effects in CHOP si-RNA intervention group were even better than in NFC-H group (P < 0.05, Fig. 3E). Compared with the control group, the expression of Bcl-2 gene in the high glucose group was significantly lower (P < 0.05); compared with the high glucose group, the expression of Bcl-2 gene in the NFC-H, NAC and CHOP si-RNA interference groups were significantly higher (P < 0.05); there was no difference between the NFC-H and NAC groups (P > 0.05), and the CHOP si-RNA interference group was better than the NFC-H group (P < 0.05, Fig. 3E).

Effects of NFC on ROS level and mitochondrial membrane potential of hippocampal neurons in high glucose

Our pilot study concluded that ROS level of hippocampal neurons peaked after 24 h in high glucose. Therefore we
Fig. 2 Effects of NFC on apoptosis of hippocampal neurons of diabetic rats. (A) Morphological photomicrographs of TUNEL-positive neurons of hippocampus. TUNEL positive staining neurons were labeled as brownish-yellow. Magnification 200×, scale bar = 500 μm; (B) Representative protein levels of CHOP, p-JNK, JNK, Caspase-3, Caspase-9 and Bcl2 from hippocampal tissues of the rats were assessed by Western blotting using specific antibodies; (C) Histogram indicating the fold changes in mRNA levels by real-time PCR compared to the respective control after normalization to GAPDH. Data are expressed as means ± SEM (n = 3 per group), *P < 0.05, **P < 0.01, ***P < 0.001
defined 24 h as high-glucose intervention time. DCFH-DA fluorescent probe test showed that the intensity of green fluorescence of cells in the high-glucose group increased significantly after 24 h whereas that in control and NFC intervention group was weak to the extent of being ambiguous. Photo analysis demonstrated that the mean intensity of fluorescence of hippocampal neuron ROS in the high-glucose group was significantly higher than that in the control group ($P < 0.01$). The mean intensity also decreased in all NFC groups, NAC group, JNK inhibitor group and CHOP si-RNA intervention group compared to that in the high-glucose group ($P < 0.01$). The effects were superior in NFC-H and NFC-M groups than in NFC-L group ($P < 0.01$). There was no significant difference between NFC-H, NFC-M, NAC, JNK inhibitor and CHOP si-RNA groups ($P > 0.05$, Fig. 4A).

JC-1 exists either as a green-fluorescent monomer or as a red-fluorescent aggregate. JC-1 fluorescence probe showed that the signals were largely located in the cytoplasm of hippocampal neurons. As seen in the figure, the cells in high glucose group exhibited stronger green fluorescence and weaker red fluorescence, indicating lower mitochondrial membrane potential; whereas cells in control group and NFC treatment group exhibited stronger red fluorescence and weaker green fluorescence, indicating higher mitochondrial membrane potential. Results of statistical analysis demonstrated that the ratio of red/green intensity of hippocampal neurons in the high glucose group was significantly lower than that in the control group ($P < 0.01$). The ratio was significantly higher in NFC-H, NAC, JNK inhibitor and CHOP siRNA intervention groups than that in the high glucose group ($P < 0.05$). There was no difference between NFC-H, NAC and JNK inhibitor groups ($P > 0.05$), and the ratio was higher in CHOP siRNA intervention group than that in NFC-H group ($P < 0.05$, Fig. 4B).

**Discussion**

The neuroprotective effects of Naofucong on multiple pathways and targets have been confirmed by experimental studies, which showed that Naofucong played a clear role in promoting learning and memory, and inhibiting neuronal apoptosis, etc.; however, the mechanism of the neuroprotective effects on hippocampal neurons in the presence of high glucose has not been elucidated. The results of this study showed that Naofucong effectively inhibited the apoptosis of hippocampal neurons induced by high glucose in vitro and in diabetic rats, and its neuroprotective effects might be related to the inhibition of CHOP/JNK/Bcl2 apoptosis pathway, and amelioration of oxidative stress and mitochondrial damage.
Clinical studies and experimental studies have shown that diabetes can lead to a decline in cognitive ability, and the abnormalities in hippocampal structure and function play a key role in the development of cognitive impairment in DM \[21\]. It has been found that the blood glucose level is closely related to the hippocampal volume and synaptic plasticity in diabetic patients and DM animal models \[19\]. In DM model, the apoptosis rate of hippocampal cells is significantly increased, and there are abnormalities in neurogenesis and synaptic plasticity \[20\]. It has been proved in vitro that long-term high glucose culture can not only decrease the activity of hippocampal neurons and increase apoptosis, but also cause abnormalities in the content and distribution of synaptic proteins \[22\]. Therefore, high glucose is a key pathological factor in the damage of hippocampal neurons. At present, the specific mechanism of high glucose induced damage of hippocampal neurons has not yet been fully elucidated, which might be related to high glucose induced oxidative stress, mitochondrial damage, inflammatory response, electrophysiological abnormalities and changes in neurotransmitters. In this experiment, hippocampal neuronal apoptosis was significantly increased in diabetic cognitive dysfunction rats; the in vitro results of TUNEL in situ detection and Annexin V/PI staining showed that high-glucose culture for 72 h could significantly increase the apoptosis rate of hippocampal neurons. These results were consistent with the results reported in the in vivo studies \[21\], and the effect of high glucose on the damage of hippocampal neurons was confirmed in vitro. The intervention of Naofucong effectively inhibited the apoptosis of hippocampal neurons in vivo and in vitro experiments.

The activation of CHOP and JNK signaling pathway is critical in the occurrence of neuronal apoptosis, and its importance has been confirmed in a variety of neural injury models. Animal experiments showed that the expression of CHOP, p-JNK, JNK, Caspase-3 and Caspase-9 was increased, while Bcl2 was significantly decreased in the hippocampus of diabetic rats with cognitive impairment; and furthermore, in vitro studies have shown that high glucose could upregulate the expression of CHOP in protein and mRNA levels, significantly increase the expression of phosphorylated JNK, and down-regulate the protein and mRNA of Bcl2, suggesting that high glucose can induce the activation of JNK and CHOP-Bcl2 apoptotic pathway in hippocampal neurons. The results showed that the addition of CHOP si-RNA or JNK inhibitors significantly reduced the apoptosis rate of hippocampal neurons and increased the ex...
pression of Bcl2 in protein and mRNA transcription level in the presence of high glucose, indicating that the inhibition of high glucose induced activation of the CHOP and JNK apoptosis pathway could significantly decrease the apoptosis of hippocampal neurons. Therefore, the mechanism of apoptosis induced by high glucose is related to the activation of CHOP and JNK apoptosis pathway. Our results showed that after Naofucong treatment, the phosphorylation level of JNK was decreased in hippocampal neurons, the expression of CHOP was down-regulated and the Bcl2 was up-regulated in protein and mRNA level, suggesting that the Naofucong might play anti-apoptotic effect by interfering with the activation of the CHOP and JNK apoptosis pathway.

The latest research showed that [23, 24] apoptosis can also aggravate the cell damage by oxidative stress and mitochondrial damage pathway. The oxidative stress also enhances the transcription of CHOP. The results of DCFH-DA fluorescence probe showed that high glucose could induce oxidative stress in hippocampal neurons. Oxidative stress inhibitor NAC significantly reduces the ROS, down-regulates the expression of CHOP in protein and mRNA transcription, and decreases the apoptosis rate of hippocampal neurons; and furthermore, JNK inhibitors and knock-down of CHOP by siRNA interference also reduce the ROS in hippocampus, suggesting that there are cross-talk pathways between oxidative stress and apoptosis, and high glucose aggravates the neuronal damage through activating the cross-talk pathways. Experimental results showed that Naofucong significantly reduced the ROS in hippocampus, indicating that the neuroprotective effect of Naofucong might be related to the inhibition of oxidative stress and the cross-talk between oxidative stress and apoptosis pathways.

In addition, mitochondrial damage is one of the mediators of apoptosis pathways. The results of JC-1 fluorescence probe showed that high glucose could induce significant decrease in the mitochondrial membrane potential of hippocampal neurons, indicating that high glucose could induce the activation of mitochondrial damage pathway. The results showed that JNK inhibitor and CHOP siRNA significantly increase the mitochondrial membrane potential of hippocampal neurons, suggesting that high glucose might aggravate the damage of hippocampal neurons through activating the cross pathway between apoptosis and mitochondrial damage pathway. This study showed that Naofucong significantly increased the mitochondrial membrane potential of hippocampal neurons, suggesting that Naofucong can play neuroprotective role by inhibiting mitochondrial damage pathway.

In summary, high glucose induces apoptosis of hippocampal neurons by activation CHOP and JNK pathway, and the neuronal injury induced by high glucose is also related with the activation of the cross pathway of oxidative stress
and mitochondrial damage. Naofucong has inhibitory effects on CHOP and JNK apoptosis pathway induced by high glucose, and also decreases the expression of ROS and increases the mitochondrial membrane potential, all of which suggest that the neuroprotective effect is related to the inhibition of JNK and CHOP pathway, as well as the activation of the cross pathway between apoptosis, oxidative stress and the mitochondrial damage pathway. There are some limitations in this study. Endoplasmic reticulum (ER) stress is recognized in the pathogenesis of diabetes, and CHOP apoptosis signaling pathways can be triggered by ER stress, which is an important downstream of ER stress. It has not been fully elucidated in this study. However, we speculate that ER stress probably plays an important role in the apoptosis induced by high glucose, and NFC can inhibit ER stress, which will be investigated in our future studies.

Conclusion

Naofucong significantly improves the cognitive function under high glucose concentration, which is related to the inhibition of CHOP and JNK apoptotic signaling pathways, and the cross pathway between apoptosis, oxidative stress and the mitochondrial damage pathway.

References