Huangdi Anxiao Attenuated High Glucose-Induced PC12 Cells Neurotoxicity via Inhibiting Apoptosis Pathway of Endoplasmic Reticulum Stress

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Research Article

Keywords: Huangdi Anxiao, Diabetic cognitive dysfunction, Neuroprotection, Apoptosis pathway of endoplasmic reticulum stress

Posted Date: December 29th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1169332/v1>

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Abstract

Background

Huangdi Anxiao (HDAX) is mainly used to treat diabetes and its complications for many years and has a remarkable curative effect. However, the improvement effect of HDAX in the diabetic cognitive dysfunction (DCD) model and the related mechanism is not clear. This study was aimed to explore the neuroprotective effects of HDAX and its possible mechanisms in DCD.

Methods

A DCD cell model was established by high glucose-induced PC12 cells, and the effect of HDAX on the cell viability was examined by MTT. Additionally, the expression of relevant genes and proteins in the apoptosis pathway of endoplasmic reticulum (ER) stress was detected.

Results

The results showed that HDAX increased cell viability, reduced GRP78, CHOP, Bax, procaspase-12, procaspase-9, procaspase-3 mRNA levels and GRP78, CHOP, Bax, Caspase-12, Caspase-9, Caspase-3 protein expressions, and decreased Bcl-2 mRNA level and protein expression.

Conclusions

These results suggested that HDAX had neuroprotective effects in the DCD cell model, which may be associated with the inhibition of the apoptosis pathway of ER stress.

Background

Diabetic cognitive dysfunction (DCD) is a neuropathy caused by sustained hyperglycemia, which is one of the serious complications of diabetes. It is mainly manifested as the decrease of learning and memory ability, accompanied by pathological changes in brain structure and neurological function, which seriously affects the quality of life [1]. Studies have shown that people with diabetes are at least twice as likely to have severe impairment of cognitive function, type 2 diabetes mellitus (T2DM) is a risk for Alzheimer's disease (AD) [2, 3]. Although a growing number of researchers have studied over the past few decades, the pathogenesis of DCD remains unclear.

Glucose is the main source of energy, which mammalian brain and its essential function for the normal brain. Recent studies have shown that high glucose is the most fundamental cause of DCD, high glucose environment can lead to increased apoptosis of nerve cells, decrease the ability of learning and memory, and eventually lead to the occurrence of DCD [4]. Besides, chronic hyperglycemia can promote the

deposition of Amyloid beta-peptide (A β), increase the sensitivity of nerve cells, and thus generate A β of neurotoxicity to cerebral microvascular endothelial cells [5]. Some experimental studies showed that high glucose-induced brain insulin resistance leads to changes similar to AD [6]. The exact mechanism of the nerve cell damage caused by high glucose is unclear, but studies have shown that glucose toxicity causes metabolic abnormalities in the following pathways: Endoplasmic reticulum (ER) stress, oxidative stress, inflammatory response, insulin resistance, etc. [7–9]. The apoptosis pathway of ER stress is hot in recent years.

The apoptosis pathway of ER stress has been increasingly studied in the occurrence and development of various diabetic complications [10]. ER stress response can be triggered by different factors, such as high glucose environment, inflammatory cytokines, the disorder of calcium balance, oxidative damage, etc. [11], through unfolded protein response (UPR) protect and restore cell function. However, when the injury cannot be recovered in time, UPR can further regulate the downstream apoptotic signaling molecules such as C/EBP homologous protein (CHOP), CysteinyI aspartate specific proteinase (Caspase), and B-cell lymphoma-2 (Bcl-2) family to induce apoptosis [12]. In recent years, to find an effective treatment for DCD, researchers have begun to try to alleviate the disease by regulating the apoptosis pathway of ER stress. Therefore, the development of appropriate drugs has become a hot spot and basic research in a clinic of DCD.

Huangdi Anxiao (HDAX) is a hospital formula from Anhui Provincial Hospital of Chinese Medicine. It is mainly used to treat diabetes and its complications for many years and has a remarkable curative effect [13, 14]. However, the improvement effect of HDAX in the DCD model and the related mechanism is not clear. We speculate that HDAX may be effective in the treatment of DCD by inhibiting the apoptosis pathway of ER stress. To elucidate the mechanism of HDAX therapy of DCD, we used high glucose to build DCD vitro model in PC12 cells, to explore the ability of HDAX in neuroprotective effects and related proteins and gens.

Materials And Methods

Materials

HDAX were mainly composed of *Coptidis Rhizoma*, *Radix Rehmanniae*, *Radix Puerariae*, *Radix Ophiopogonis*, *Eriobotryae Folium*, and *Radix Notoginseng*, which were purchased from Anhui Provincial Hospital of Chinese Medicine. Notoginsenoside R1, ginsenoside Rg1, puerarin, acteoside, berberine, coptisine, and palmatine hydrochloride were purchased from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). 4-phenylbutyric acid (4-PBA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibody of GRP78, CHOP was purchased from Affinity Bioscience (1:1300, USA); Caspase-12, Caspase-9, Caspase-3 were obtained from Bioss (1:1000, Beijing, China); Bax, Bcl-2 was provided by Bioworld (1:1000, Nanjing, China).

Preparation of HDAX

HDAX solution was prepared by boiling these six components *Coptidis Rhizoma* (6 g), *Radix Rehmanniae* (10 g), *Radix Puerariae* (10 g), *Radix Ophiopogonis* (8g), *Eriobotryae Folium* (12 g), and *Radix Notoginseng* (5 g) in water. Adding and refluxing 10-fold water for 1.5 h, then adding 8-fold water and repeated before for 1.0 h. Then, collecting the solution and concentrating to 10 mg/mL, and stored at 4°C.

Ultra-performance liquid chromatography (UPLC) analysis of HDAX

Puerarin, acteoside, coptisine, palmatine hydrochloride, berberine, notoginsenoside R1, and ginsenoside Rg1 in HDAX were measured by UPLC. An Acquity BEH C18 analytical column coupled with a column filter at 30°C. Gradient elution with 0.05% (v/v) phosphoric acid in distilled water (A) and acetonitrile (B): 3%-15% B for 0-6 min, 15%-27% B for 6-7 min, 27%-27% B for 7-13 min, 27%-70% B for 13-15 min, 70%-3% B for 15-16 min. The flow rate is 0.2 mL/min and injection volumes is 1 µL. Finally monitored at 203 nm and 260 nm.

Preparation of HDAX containing serum

Twenty rats were divided into two groups randomly: Control group, HDAX group, 10 rats in one group. Referring to the method of the previous experiment(Cai et al., 2018), HDAX was prepared into the solution at a dose of 10.5 g/kg (7 times of the clinical equivalent dose), the drug was given by gavage of 10 mL/kg for 5 d. The control group was given an equal volume of normal saline. After the last administration, each group was collected blood samples under the abdominal aorta under aseptic conditions, centrifugation for 15 min at 3000 r/min, serum samples were collected, inactivated for 56°C for 30 min, and stored at -20°C for later use.

Cell culture and treatments

PC12 cells were cultured in Dulbecco modified Eagle medium (DMEM, Thermo Fisher Scientific, Inc., Waltham, USA) in an incubator containing 5% CO₂ at 37 °C. They were digested by 0.25% pancreatin for passage when the cells reached the confluence rate of above 80%.

Based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) experiment, the PC12 cells were assigned to the following treatment groups: Control, Model, 15% HDAX containing serum group (HDAX), 4-PBA inhibitor group (4-PBA), and 15% HDAX containing serum+4-PBA inhibitor group (HDAX+4-PBA). The model group was incubated with 100 mM glucose for 48 h to establish the DCD cell model. HDAX+4-PBA group were incubated with 15% HDAX containing serum and 5 µM 4-PBA inhibitor, HDAX, and 4-PBA groups were given 15% HDAX containing serum and 5 µM 4-PBA inhibitor, respectively. establishing the DCD cell model after 24 h.

Cell viability

Add MTT solution (20 µL, 5 mg/mL) to each hole and incubated, then solubilize the precipitated dye with DMSO. After dissolved for 10-15 min, measured the absorbance at 490 nm with a microplate reader (318C+, Shanghai, China).

The observation of inverted microscope

The cells were seeded onto a six-well plate at a density of 1×10^6 cells/m. The cells were treated with glucose (50, 75, 100, 125, 150 mM) for 48 h. When the cell growing time and density meet the requirement, we selected the clear field of views to observe the morphology and structure of the cells by inverted microscope.

Hoechst 33342 staining

PC12 cells in the logarithmic growth stage were treated according to different groups. After fixation with 4% paraformaldehyde for 10 min, 1 mL Hoechst 33342 dye diluent was added to each hole and was treated at 37°C incubator for 20-30 min, then PBS washed for 2-3 times about 1-2 min. Observing and photographing by fluorescence microscopy (Olympus IX71).

Quantitative real-time PCR (RT-qPCR)

Total mRNA of cells was extracted by Trizol reagent (Ambion, USA). The complementary cDNA was synthesized with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Massachusetts, USA). According to SYBR (TOYOBO, Japan), mRNA was detected by 7500 real-time PCR (Applied Biosystems, Foster City, CA). All target gene primers were designed with the comparative Ct ($2^{-\Delta\Delta C_t}$) (cycle threshold) method which is listed below.

Table 1

Gene		Sequences(5'→3')
β-actin	Forward	TTGATTTGGCTGGTAGAA
	Reverse	ATGGCAGAAGATTGAGAA
GRP78	Forward	CGGAGGAGGAGGACAAGAAGGAG
	Reverse	ATACGACGGTGTGATGCGGTTG
CHOP	Forward	CCTCGCTCTCCAGATTCCAGTCAG
	Reverse	TCTCCTGCTCCTTCTCCTTCATGC
Bax	Forward	CCAGGACGCATCCACCAAGAAG
	Reverse	GCTGCCACACGGAAGAAGACC
Bcl-2	Forward	ACGGTGGTGGAGGAACTCTTCAG
	Reverse	GTGCAGATGCCGGTTCAGGTAC
procaspase-12	Forward	AGGGAATCCAGAGCACAGAA
	Reverse	GCCTTCCTTCTCCATCACTG
procaspase-9	Forward	CCAGGCAGCTGGTCATAGAT
	Reverse	CCACTGCTTTGCAAGAATGA
procaspase-3	Forward	ACAGAGCTGGACTGCGGTAT
	Reverse	TAGTAACCGGGTGCGGTAGA

Western blot analysis

Total protein was extracted from the cell, quantified by BCA kit (Beyotime, China), separated by 10%-12% SDS-PAGE electrophoresis, transferred, sealed, and incubated with different primary antibodies overnight at 4°C on NC membranes. Then, the second antibody IgG (1:20,000; ZSGB-BIO, Beijing, China) combine with the primary antibody. Protein expressions were detected by ECL chemiluminescence and exposure by a gel imaging device (ProteinSimple, CA, USA).

Statistical analysis

The experiment data were expressed as the means±standard deviation (S.D.) ($\bar{x}\pm s$) and analyzed statistically using *SPSS 24.0* software (SPSS, Chicago, IL, USA). The differences in the different groups were detected by the one-way ANOVA analysis, which is considered statistically significant when $P\text{-value}<0.05$.

Results

Qualitative analysis of HDAX

Puerarin, acteoside, coptisine, palmatine hydrochloride, berberine, notoginsenoside R1, and ginsenoside Rg1 in HDAX were measured by UPLC, respectively (Figure 1 and Figure 2).

Glucose concentration screening

PC12 cells were cultured in different densities of glucose (50, 75, 100, 125, 150 mM) for 24, 48, and 72 h, the mannitol balances osmotic pressure between different groups. As shown in Figure 3, Compared with the Control group, when the concentration of building for 100 mM and 125 mM, 48 h, the cells survival rate is close to 50%. But when the mannitol concentration was 125 mM, the cell survival rate was affected ($P<0.05$). Therefore, according to the MTT results and combined with relevant references, the glucose concentration of 100 mM for 48 h was selected as the suitable experimental condition for the establishment of the DCD cell model in PC12 cells for the subsequent experiment.

Effects of glucose on cell morphology

As shown in Figure 4, After 48 h of culture with different concentrations of glucose, cells in the Control group showed regular morphology, fusiform or polygon, interwoven into a network, adherent to the wall, and good refractive index. With the increase of glucose concentration, the high-glucose injury group was significantly damaged in cell morphology, with different degrees of shrinkage, even shedding, and the intercellular space was increased.

Effects of glucose on cell apoptosis

To further determine whether high glucose-induced PC12 cell injury and promoted apoptosis, Hoechst 33342 fluorescence staining was used to evaluate the apoptosis of PC12 cells. As shown in Figure 5, after cultured for 48 h with diverse concentrations of glucose, the cells in Control group showed diffuse and uniform low-density fluorescence. With the increase of glucose concentration, some of the cells in the high-glucose injury group showed high-density fluorescence, and the nucleus appeared to be condensed and fragmented.

HDAX concentration screening

The experiment was divided into seven groups: Control group, different concentrations of HDAX containing serum groups (5%, 10%, 15%, 20%, 25%, 30%), each group respectively incubated 24 hours. As shown in Figure 6, compared with Control group, cell viability had no significant difference when the concentration of HDAX containing serum was 5%-15% ($P>0.05$), When the concentration of HDAX containing serum was 20%, the cell viability began to decline; when the concentration was 30%, the cell viability decreased significantly ($P<0.05$), so 5%, 10%, and 15% dose groups of HDAX containing serum were selected for the next experiment. After treated with high glucose, the cell viability of the 5%, 10% and 15% dose groups of HDAX containing serum was significantly higher ($P<0.05$, $P<0.01$), among which the 15% dose group of HDAX containing serum had the best effect.

Effects of HDAX on relevant mRNA levels in apoptosis pathway of ER stress on DCD cell model

To further explore the effect of the apoptosis pathway of ER stress, we used 4-PBA, an ER stress inhibitor, separately or cotreat with HDAX. Compared with Control group, the mRNA levels in Model group were significantly increased ($P<0.01$), and Bcl-2 was significantly decreased ($P<0.01$). After treatment, the mRNA levels in the HDAX group, 4-PBA group, and HDAX+4-PBA group were significantly decreased ($P<0.01$), and Bcl-2 was significantly increased ($P<0.01$), compared with Model group.

Effects of HDAX on relevant protein expression in apoptosis pathway of ER stress in DCD in cell model

After separating or cotreating with 4-PBA, compared with Control group, protein expression was significantly increased ($P<0.01$) and Bcl-2 protein was significantly decreased in Model group ($P<0.01$). After treatment, compared with Model group, protein expressions were significantly decreased in HDAX group, 4-PBA group, and HDAX+4-PBA group ($P<0.05$, $P<0.01$), the expression of Bcl-2 was significantly increased ($P<0.05$, $P<0.01$).

Discussion

Diabetic cognitive dysfunction (DCD) is a severe diabetes-related complication in the central nervous system [16]. Recent studies have shown that high glucose aggravates neurochemical and structural abnormalities in the brain, which is the most fundamental cause of DCD. Even in non-diabetic patients, impaired glucose tolerance is also an important risk factor for cognitive dysfunction [17]. High glucose can induce neuronal apoptosis and cognitive dysfunction by activating microglial inflammatory response, contributing to the occurrence and development of diabetic encephalopathy and neurodegenerative diseases [18]. It can also change the permeability of blood-brain barrier, thus accelerating the occurrence of cognitive impairment in diabetic patients [1]. As the pathological basis of DCD, it is the research direction of this experiment. In addition, previous studies have shown that HDAX is effective in the treatment of the rat model of DCD [19]. In this experiment, we further study the neuroprotective mechanism of HDAX in the DCD cell model.

First, we took PC12 cells as the main research object, which simulated high glucose and induced neurotoxicity. MTT assay, inverted microscope, and Hoechst 33342 fluorescence staining showed that cell viability significantly reduced with 100 mM high glucose treatment for 48 h, changed cell morphology, and promoted cell apoptosis. These results indicated that high glucose 100 mM induction for 48 h was the suitable experimental condition for PC12 cells to establish a high glucose injury model in vitro, which was consistent with the research results of other scholars [20, 21].

Then, we explored the therapeutic effect of HDAX in DCD. HDAX is rooted in the classical prescription "Xiao-Ke Formula", which is mainly composed of *Coptidis Rhizoma*, *Radix Rehmanniae*, *Radix Puerariae*, *Radix Ophiopogonis*, *Eriobotryae Folium*, and *Radix Notoginseng*. It has been used in the treatment of diabetes and its complications for several years, but it has rarely been studied in the research on DCD. We reanalyzed the HDAX pharmacological formula from the perspective of modern pharmacological research. In this prescription, Berberine, the main component of *Coptidis Rhizoma*, can ameliorate rats model of combined Alzheimer's disease and type 2 diabetes mellitus via the suppression of ER stress [22]. Studies in *Radix Ophiopogonis* showed that it reducing cell apoptosis, protecting islets, and improving insulin resistance [23]. *Panax notoginseng* saponins are the major active ingredients of *Radix Notoginseng*, which can keep neurons from oxidative stress damage, ameliorate learning and memory deficits in AD rats [24]. Therefore, we proposed a hypothesis that HDAX can improve DCD. We studied the neuroprotective effect of HDAX on DCD for the first time in vitro. In this experiment, we proved that HDAX can improve the cell vitality of the DCD cell model and exert its neuroprotective effect.

Next, we further explored whether the mechanism of HDAX protecting neurons is related to the inhibition of the apoptosis pathway of ER stress. We selected the related proteins and genes in this pathway for comparison, and the results were consistent with our expectations. Glucose regulated protein 78 (GRP78) was a symbolic protein and protective factor of ER stress and had the function of protecting the endoplasmic reticulum under stress [25]. CHOP was extensively induced and expressed under ER stress, and finally induces apoptosis through the mitochondrial pathway [26]. Bcl-2-associated X protein (Bax) was a pro-apoptotic protein that promoted the occurrence of apoptosis, and Bcl-2 was an anti-apoptotic protein, inhibited the occurrence of apoptosis. They usually existed in the form of heterodimers and jointly regulated the process of cell apoptosis [27]. Caspases were the core component of the apoptosis reaction. Caspase-12 was a specific apoptosis mediator in ER stress-mediated apoptosis pathway. Under normal circumstances, Caspase-12 bonded to ER membrane or formed a hetero with tumor necrosis factor receptor-associated factor 2 inactive. However, in the case of the excessive ER stress response, Caspase-12 activates downstream molecules Caspase-9 and Caspase-3 [28]. Caspase-3 was an important protease for the executive function of apoptosis. It was located downstream of the Caspase cascade and was considered to be the endpoint of apoptosis [29]. In this experiment, Our findings showed that HDAX can improve the relevant protein expression and mRNA levels after treatment. Moreover, treatment with 4-phenylbutyric acid (4-PBA), as well as cotreatment with HDAX and 4-PBA, significant inhibition of cell damage, which further demonstrated that the potential mechanism of HDAX to improve DCD by restraining the apoptosis pathway of ER stress.

Conclusions

Our findings suggest that HDAX was confirmed with a protective effect on DCD models, which might be associated with the inhibition of the apoptosis pathway of ER stress. However, there still needs deeper exploration. These data may help to explain the potential neuroprotective mechanism of HDAX on DCD.

Abbreviations

HDAX: Huangdi Anxiao; DCD: Diabetic cognitive dysfunction; ER: endoplasmic reticulum; AD: Alzheimer's disease; T2DM: Type 2 diabetes mellitus; A β : Amyloid beta-peptide; UPR: Unfolded protein response; PBS: Phosphate-buffered saline; Caspase: CysteinyI aspartate specific proteinase; Bcl-2: B-cell lymphoma-2; CHOP: C/EBP homologous protein; Bax: Bcl-2-associated X protein

Declarations

Acknowledgments

This study was supported by the National Natural Science Foundation of China (81574040 and 81873351) and the Key Project Foundation of Support Program for the Excellent Young Faculties in Universities of Anhui Province in China (grant no. gxyq ZD2018053).

Authors' Contributions

BC, GW, ZF and TY designed the study. TY, WX, PZ, NS, YW, TW, and HS performed the experiments. TY, WX and GH wrote the manuscript. All authors approved the paper to be published.

Availability of data and materials

The data will be made available upon reasonable request.

Ethics approval and consent to participate

The study protocol was approved by the Animal Manage[1]ment Center of the Anhui University of Chinese Medicine,China.

Consent for publication

All authors agree to publish the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Peak chromatograms of HDAX at 203 nm. (A) Mixed standard chromatogram (1. Notoginsenoside R1, 2. Ginsenoside Rg1), (B) HDAX chromatogram.

Figure 2

Peak chromatograms of HDAX at 260 nm. (A) Mixed standard chromatogram (3. Puerarin, 4. Acteoside, 5. Coptisine, 6. Palmatine hydrochloride, 7. Berberine). (B) HDAX chromatogram.

Figure 3

Effects of different times and concentrations of glucose on cell viability. (A) PC12 cells viability in 24 h, 48 h, and 72 h. (B) PC12 cells viability in different mannitol concentrations. The values are presented as the means±S.D. of 3 individual experiments. Compared to the Control, * $P<0.05$, ** $P<0.01$.

Figure 4

Morphological effects of different glucose concentrations on PC12 cells (100×). (A) Control; (B) 50 mM glucose (C) 75 mM glucose; (D) 100 mM glucose; (E) 125 mM glucose; (F) 150 mM glucose.

Figure 5

Apoptosis of PC12 cells was observed under fluorescence microscope (200×). (A) Control; (B) 50 mM glucose; (C) 75 mM glucose; (D) 100 mM glucose (E) 125 mM glucose; (F) 150 mM glucose.

Figure 6

HDAX concentration screening. (A) Effects of different concentrations of HDAX containing serum on the survival rate of PC12 cells. (B) Effect of different concentrations of HDAX containing serum on the

survival rate of cell model. The values are presented as the means±S.D. of 3 individual experiments. Compared to the Control, ^{**}*P*<0.01; compared to the Model, [#]*P*<0.05, ^{##}*P*<0.01.

Figure 7

Effect of HDAX on mRNA levels of GRP78, CHOP, Bax, Bcl-2, procaspase-12, procaspase-9, and procaspase-3 in DCD cell model. The values are presented as the means±S.D. of 3 individual experiments. Compared to the Control, ^{*}*P*<0.05, ^{**}*P*<0.01; compared to the Model, [#]*P*<0.05, ^{##}*P*<0.01.

Figure 8

Effect of HDAX on the protein expression of GRP78, CHOP, Bax, Bcl-2, Caspase-12, Caspase-9, and Caspase-3 in DCD cell model (A-G). The values are presented as the means±S.D. of 3 individual experiments. Compared to the Control, * $P<0.05$; ** $P<0.01$; compared to the Model, # $P<0.05$; ## $P<0.01$.