Protective effects of Huanglian Wendan Decoction against cognitive deficits and neuronal damages in rats with diabetic encephalopathy by inhibiting the release of inflammatory cytokines and repairing insulin signaling pathway in hippocampus

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[ABSTRACT] Huanglian Wendan decoction (HLWDD) has been used for the treatment of symptom of “Re”, one of major causes in diabetes and metabolic disorders, according to the theory of traditional Chinese medicine. The present study aimed at investigating the cerebral protective effects of HLWDD on diabetic encephalopathy (DE), one of the major diabetic complications. The effects of HLWDD and metformin were analyzed in the streptozocin (STZ) + high-glucose-fat (HGF) diet-induced DE rats by gastric intubation. In the present study, the effects of HLWDD on cognition deficits were investigated after 30-day intervention at two daily dose levels (3 and 6 g·kg⁻¹). To explore the potential mechanisms underlying the effects of HLWDD, we detected the alterations of neuronal damages, inflammatory cytokines, and impaired insulin signaling pathway in hippocampus of the DE rats. Based on our results from the present study, we concluded that the protective effects of HLWDD against the cognitive deficits and neuronal damages through inhibiting the release of inflammatory cytokines and repairing insulin signaling pathway in hippocampus of the DE rats.

[KEY WORDS] Diabetic encephalopathy; Insulin signaling pathway; Cognition; Huanglian Wendan decoction


Introduction

Diabetic encephalopathy (DE) is one of the most widespread diabetic complications [1], with similar symptoms and behaviors of Alzheimer’s disease (AD). Recognized as an analogous disease of diabetes and AD, DE not only shows chronic hyperglycemia and insulin resistance in periphery, but also presents cognitive deficits and pathological changes in brain. Being discovered in 1922, there is still no clear answer to its pathogenesis. Chronic high-glucose-fat (HGF) diet combined with streptozocin (STZ) injection causes hyperglycemia which is considered as an initiator in the development of DE [2]. Reports declare that chronic hyperglycemia not only shows inhibitions on glucose metabolism in brain [3], but also alters neuronal damages, cognitive function [4], insulin signaling pathway [5], and inflammatory response [6] in the brain of the rats. All these damages are linked by the network of insulin signaling pathway.

According to recent studies [7-9], dysfunction of insulin signaling pathway, presented as insulin resistance generally, has been observed in brains of DE rats which may be partly responsible for DE pathogenesis. Evidences support that the impairment of insulin signaling pathway may also be responsible for the occurrence of cognitive deficits and neuronal damages [10-11] via regulation of cellular function. This has been confirmed by a study with intranasal...
administration of insulin that prevents cognitive decline and other cerebral damages in an animal model of DE [12]. Researches also show that an anti-diabetic agent protects the mouse brain from defective insulin signaling and improves Alzheimer-like pathology in the brain [13]. All these studies indicate that the impaired insulin signaling pathway in brain may be involved in the DE process.

There are many factors leading to the dysfunction of insulin signaling pathway. Inflammatory cytokines have also been identified as the major etiology factors for dysfunction of insulin signaling pathway [14-15]. Inflammation is one of defense mechanisms of body induced by stress, infections, and injuries. As an inflammatory disease, diabetes causes inflammatory response over the whole body, including the brain, after damaged by hyperglycemia. High levels of inflammatory cytokines are detected in brain of diabetic rats. Recent studies also report the application of anti-inflammatory drugs, such as ibuprofen, has also shown improvements in DE rats [16]. The inflammatory cytokine TNF-α has been reported to trigger the phosphorylation of Ser307 in insulin receptor substrate-1 (IRS-1), which blocks insulin signaling pathway [17] by promoting multipotential signal transduction cascades, including the activation of c-Jun NH2-terminal kinase (JNK). However, the impaired insulin signaling pathway would lose the controlling of inflammatory response which leads to more release of inflammatory cytokines. Therefore, the present study focused on the improvement of Huanglian Wendan decoction (HLWDD), a Chinese traditional prescription, on inflammatory response, insulin signaling pathway, and their relationship in DE.

HLWDD is consisted of root and rhizome of Coptis deltoidea C. Y. Cheng & P. K. Hsiao (family: Ranunculaceae), cortex of Bambusa textilis McClure (family: Poaceae), caulis of Pinellia ternata (Thunb.) Makino (family: Araceae), fructus of Citrus aurantium L. (family: Rutaceae), pericarpium of Citrus reticulata Blanco (family: Rutaceae), dried sclerota of Poria cocos (Schw.) Wolf. (family: Polyporaceae), root and rhizome of Glycyrrhiza uralensis Fisch. (family: Leguminosae), root and rhizome of Zingiber officinale Roscoe (family: Zingiberaceae). It was first recorded in “Liu-yin-tiao-bian”, a medical book written in Qing dynasty. While used for the traditional treatment of diabetes [18] and its complications [19] by its hypoglycemic action, the exact effects of HLWDD on DE remain enigmas. Recent clinical studies demonstrate that HLWDD improves insulin resistance [20] and inflammation [21] in peripheral system. However, there is little or no report on the relation between HLWDD and anti-inflammation or insulin signaling in brain. Our study demonstrated its benefits in DE and explored its underlying mechanism in brain using a rat DE model.

Materials and Methods

Materials

Streptozocin (STZ) was purchased from Sigma (St. Louis, MO, USA). Metformin hydrochloride was obtained from hospital produced by Jiangsu Suzhong Pharmaceutical Group Co., Ltd. Other reagents and kits used in the present study and their sources were as follows: Glucose testing kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) ELISA kits (R&D, Minneapolis, MN, USA), RNA isolator total RNA extraction reagent (Vazyme, Nanjing, China), Easy Script® First-Strand cDNA Synthesis Super Mix (Trans Gen Biotech Co., Ltd., Beijing, China), and Trans Start® Green qPCR Super Mix UDG (Trans Gen Biotech Co., Ltd., Beijing, China). Primers used in the present research were synthesized by GenScript (Nanjing) Co., Ltd. (Nanjing, China).

Preparation of herbal extracts

The herbal materials were bought from Anhui Welлон Chinese Medicine Yinpian Co., Ltd. in Anhui (License Number: Wan 20100112) and identified by Prof. Qin Min-Jian (Department of Medicinal Plants, China Pharmaceutical University, Nanjing, China). The voucher specimens, whose voucher numbers were CPU-HWD-RC-140113, CPU-HWD-CB-140113, CPU-HWD-CP-140113, CPU-HWD-FC-140113, CPU-HWD-PC-140113, CPU-HWD-SP-140113, CPU-HWD-RG-140113, and CPU-HWD-RZ-140113, were all deposited in Department of Natural Medicinal Chemistry, China Pharmaceutical University.

All of herbal materials were dried, crushed, and mixed according to the weight ratio of 4 : 4 : 4 : 4 : 6 : 3 : 2 : 2 in Liu-yin-tiao-bian before a 30-min soak. Then the final herbal extracts were obtained after two 30-min reflux extractions in water and filtrated. The yield of HLWDD was 32.9% and it was stored at −20 ºC until use.

Experiment design

Male Sprague Dawley (SD) rats weighing 200~220 g (License Number: SCXK2009-002) were purchased from the Experimental Animal Center in Jiangsu (Nanjing, China). The rats were housed in a standard animal room under well controlled conditions in a 12 h/12 h light/dark cycle and provided with free access to food and tap water. After one week of acclimation, the rats were then randomly divided into five groups (10/group): normal group, DE (diabetic encephalopathy) group, metformin (0.2 g·kg⁻¹) group, HLWDD-3 (3 g·kg⁻¹) group, and HLWDD-6 (6 g·kg⁻¹) group.

Nextly, after fed with high-glucose-fat (HGF) diet (formula: 15% lard, 10% sucrose, 3% cholesterol, 3% sodium chloride, and 69% standard rat feed) for 50 days, all the rats, except for the normal group (fed with standard rat feed), were intraperitoneally injected with a single dosage of 35 mg·kg⁻¹ of STZ. Fasting blood-glucose (FBG) was measured to assess whether the rat model of diabetes was successfully established as described previously [22]. Metformin hydrochloride (0.2 g·kg⁻¹), and HLWDD (3 and 6 g·kg⁻¹) were given daily by gavage for 30 days about 10 mL·kg⁻¹ for a rat while the normal and DE groups received the same volume of saline (Fig. 1). HGF was still given to each group.
Measurements of body weight, FBG, triglyceride (TG), and total cholesterol (TC)

Body weight of the rats was recorded. Blood was obtained from caudal veins and centrifuged at 3,000 r·min⁻¹ for 15 min at 4 ºC. The supernatant was separated as serum and FBG was measured by a biochemical kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) using the glucose oxidase method. TG and TC in serum were also measured by biochemical kits (both from Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions.

Learning and memory tests

Morris water maze test was performed at the end of drug administration to determine the learning and memory abilities of the rats (n = 10). The test was carried out twice each day in a 120-cm diameter water pool which was divided into four quadrants and placed in a 21-cm high platform in center of one quadrant. The pool was filled with water for 22-cm deep at 23–27 ºC, with four marks placed on the wall. 90 seconds were left for each rat to find the platform which was 1 cm below the water. After the rats were placed into the pool, the time was recorded as escape latency when the rat reached the platform. If a rat failed to find platform in 90 s, it was guided. Then 30 s were left for each rat on the platform to memorize the environment around.

Following a five-day training period, a probe trail test was performed to detect the learning and memory of the rats on the sixth day. Simultaneously, the platform was removed. The times of rats spending on the target quadrant and the frequencies of rats passing the platform were recorded by EthoVision XT 8.0 (ZS-001 Morriss, ZS Dichuang Technological Development Co., Ltd., Beijing, China). All PCR primers were designed from the published sequence data and validated by NCBI database.

Histological analysis

Three rats of each group were subjected to a perfusion from heart to the whole body with 150 mL of cold 0.1 mol·L⁻¹ phosphate buffer solution (pH = 7.4) and 100 mL of cold 4% paraformaldehyde solution in 30 min. The brains were then removed and preserved in 10% formaldehyde solution. After dehydration, 4 µm-thick serial coronal paraffin sections were prepared using a freezing microtome.

H&E staining and Congo red staining were performed on the sections according to Li’s method [23] and the protocols of the manufacturer (Sigma Accustain amyloid staining kit, St. Louis, MO). After dyed, washed, dehydrated and coverslipped, the pictures were captured from regions of CA1 in hippocampus under an optical microscope. After H&E staining, as well as the expression of amyloid protein in hippocampus after Congo red staining, the integrated neurons in CA1 region of hippocampus were counted and compared with an Image-pro Plus 6.0 software (Media Cybernetics, Inc., USA).

Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from hippocampus with Trizol reagent and reverse transcribed to cDNA directly using Reverse Transcription Reagents kit (Trans Gen Biotech Co., Ltd., Beijing, China). All PCR primers were designed from the published sequence data and validated by NCBI database.

Histological analysis

The activation of insulin signaling pathway was detected by Western blotting analysis. 0.1% phenylmethylsulfonyl fluoride lysis buffer was used to extract total proteins from hippocampus. Then 5 × concentrated loading buffer (0.5 mol·L⁻¹ Tris-HCl 2.5 mL, DTT 0.39 g, SDS 0.5 g, BPB 25 mg, glycerinum 2.5 mL) was added into the protein extracts. Finally, the compound was heated at 100 ºC for 10 min. After quantified for the total protein concentration, the protein extracts were stored at −20 ºC until analysis.

The proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes. Then the membranes were blocked with 5% nonfat dry milk in TBST (Tris-buffered saline Tween-20) for 2 h at room temperature with a mild shaking. The PVDF membranes were incubated with primary antibodies of p-JNK1/2/3 at T183/Y185 (1 : 500, Bioworld), insulin receptor (InsR) primer sequences were: forward, 5’-TGACAATGAGGAATGTGGGGAC-3’, reverse, 5’-GGGC AAACTTTCGACAATGACTG-3’. Insulin receptor substrate 1 (IRS-1) primer sequences were: forward, 5’-GATACCGATG GCTTCTCAGACG-3’, reverse, 5’-TCGTTCCTCATAACT CCAAGGCC-3’. GAPDH primer sequences were: forward, 5’-GGGTGTGAACACGAGAAT-3’, reverse, 5’-GGAAG AATGGGAGTTGCTGT-3’. The real-time qPCR was performed using AceQTM qPCR SYBR® Green Master Mix and the results were analyzed with Bio-Rad CFX Manager 3.0 software, with the data being converted to 2⁻^ΔΔCT.
min thrice, the membranes were incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (h + l) secondary antibody (1: 5 000, Cell Signaling Technology, Inc., Shanghai, China) at room temperature for 2 h. After washing with TBST as above, the PVDF membranes were developed using enhanced chemiluminescence reagents and exposed to Kodak X-OMAT blue film (Kodak) in a dark room. The results were imaged and quantified using Image-J Launcher software (National Institutes of Health, USA).

**Quantitative HPLC analysis**

For HPLC analysis, 3 mg of HLWDD was dissolved in the 1 mL of double distilled water and filtered through a 0.2-μm nylon membrane prior to injection onto HPLC. The berberine standard solution was prepared at a dose of 0.5 mg (10 mg·mL⁻¹). Quantitative HPLC analysis was performed on an Agilent 1200 series HPLC system (Agilent Technologies, Inc., Nanjing, China) equipped with a HPLC quaternary pump (G13311A), auto sampler (G1329A), degasser (G1322A), UV detector (G1315D), and column oven (G1316B) and controlled by EZchrome software (Agilent Technologies, Inc., Nanjing, China) which was used for data analysis and processing. Separation was carried out on a RP-C 18 column (4.6 mm × 250 mm; particle size 5 μm; Agilent, Zorbax Eclipse plus) with column oven temperature of 25 °C using a gradient elution of eluents B (water) and C (methanol). The gradient program was as follows: 0–5 min, 20% C; 5–10 min, 50% C; and 10–15 min, 70% C. The flow rate of 0.8 mL·min⁻¹ was maintained throughout the analysis and the injection volumes ranged from 2.0 to 10.0 μL. The detection was performed at 345 nm.

**Statistical analysis**

The data were expressed as means ± SEM (standard error of mean). The comparisons were made by one-way analysis of variance (ANOVA) followed by Turkey’s test using SPSS 17.0 software. *P < 0.05 was considered statistically significant.

**Table 1 Effects of HLWDD on body weight, FBG, TG and TC in DE rats (Mean ± SEM, n = 10)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (/g·kg⁻¹)</th>
<th>Body weight (/g)</th>
<th>FBG (/mg·dL⁻¹)</th>
<th>TG (/mg·dL⁻¹)</th>
<th>TC (/mg·dL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>422.0 ± 32.6</td>
<td>81.7 ± 2.3</td>
<td>64.9 ± 6.3</td>
<td>54.3 ± 4.5</td>
</tr>
<tr>
<td>DE</td>
<td>—</td>
<td>276.83 ± 23.2</td>
<td>452.8 ± 43.0</td>
<td>364.4 ± 29.9</td>
<td>666.1 ± 69.0</td>
</tr>
<tr>
<td>Metformin</td>
<td>0.2</td>
<td>338.0 ± 28.4</td>
<td>200.3 ± 33.0</td>
<td>70.5 ± 8.5</td>
<td>135.3 ± 12.5</td>
</tr>
<tr>
<td>HLWDD</td>
<td>3</td>
<td>332.0 ± 24.9</td>
<td>230.6 ± 40.6</td>
<td>233.3 ± 36.0</td>
<td>248.9 ± 31.5</td>
</tr>
<tr>
<td>HLWDD</td>
<td>6</td>
<td>339.0 ± 20.8</td>
<td>215.7 ± 31.5</td>
<td>207.6 ± 32.5</td>
<td>215.3 ± 16.8</td>
</tr>
</tbody>
</table>

(DE, diabetes encephalopathy. *P < 0.05, **P < 0.01 vs Normal; *P < 0.05, **P < 0.01 vs DE)

**Results**

**Effects of HLWDD on body weight, FBG, TG, and TC**

Body weight, FBG, TG and TC were measured to confirm the effects of HLWDD on diabetes (Table 1) at the end of the drug administration. All drug treated groups, including 0.2 g·kg⁻¹ of metformin and 3 and 6 g·kg⁻¹ of HLWDD groups, showed increase in body weight (*P < 0.01) and decreases in FBG (*P < 0.01 or *P < 0.05), TG (*P < 0.01 or *P < 0.05) and TC (*P < 0.01), compared with the DE model group.

**Effects of HLWDD on Morris water maze testing results**

The results of Morris water maze testing are presented in Fig. 2. After a 5-day training period, the latency of each group was decreased (Fig. 2C). On the fourth day, the DE group exhibited obvious longer latency (*P < 0.05) compared with normal group. And the improvement of the metformin group rats was significant (*P < 0.05), compared with the DE group. At the fifth day, the results showed remarkable difference (*P < 0.01) between the DE group and the normal group. However, the drug treated groups (*P < 0.01 or *P < 0.01), especially metformin group (*P < 0.01), displayed significant reduction in the latency, compared with the DE group.

In the probe trail test, the fewer platform crossing (Fig. 2A, *P < 0.05) and less time spent in the target quadrant (Fig. 2B, *P < 0.01) demonstrated a defective cognition function in the DE group rats, compared to that in the normal rats. The rats in the drug treated groups (*P < 0.05 or *P < 0.01) showed improvements of cognitive function by enhancing the
Fig. 2  Effects of HLWDD in Morris water maze test in the DE rats. The frequency of platform crossing (A), the time spent in target quadrant (B) on the sixth day and the latency time recorded when rats found the platform in five training days (C) were measured (Mean ± SEM, n = 10. #*P < 0.05, ##P < 0.01 vs Normal; *P < 0.05, **P < 0.01 vs DE)

Effects of HLWDD on the release of proinflammatory cytokines in hippocampus

Three proinflammatory cytokines, TNF-α, IL-6 and IL-1β, were quantified in hippocampus (Figs. 3A, B, and C). And marked changes (P < 0.05 or P < 0.01) between groups indicated that treatments of metformin, HLWDD (3, and 6 g·kg\(^{-1}\)) alleviated the release of proinflammatory cytokines (P < 0.05 or P < 0.01), compared with the DE group.

Effects of HLWDD on histopathology in hippocampus

Cellular morphology could be visualized by H&E staining. There were seriously damaged cells emerged in hippocampus of the DE rats (Fig. 4). Then the relative density of integrated neurons at CA1 region of hippocampus was counted and analyzed. Apparently, the significant neuron loss was emerged in the DE group (P < 0.01) compared with the normal rats. The rats in metformin and HLWDD groups showed increases in neuron density, compared with the DE group (P < 0.05 or P < 0.01).
Effects of HLWDD on neuron density (400 ×) in CA1 region of hippocampus in HE staining. The relative neuron density in CA1 region of hippocampus was statistical analyzed. The arrow represents apoptotic neuron (Mean expression of normal group = 1. Data were noted as Mean ± SEM, n = 3. # P < 0.05, ## P < 0.01 vs Normal; * P < 0.05, ** P < 0.01 vs DE)

Effects on amyloid deposition by Congo red staining

Congo red staining was used to present the amyloid deposition in hippocampus (Fig. 5). The DE group showed significantly amyloid protein accumulation (P < 0.01, compared with the normal controls), which was reversed by the treatments of 0.2 g·kg⁻¹ metformin and 3 and 6 g·kg⁻¹ of HLWDD (P < 0.05, compared with the DE group).

Effects of HLWDD on the expression of mRNAs

The expressions of InsR (Fig. 6A) and IRS-1 (Fig. 6B) mRNAs were significantly decreased in the DE group, compared with the normal controls (P < 0.05, P < 0.01). However, the treatments of metformin (0.2 mg·kg⁻¹) and HLWDD (3 and 6 g·kg⁻¹) obviously enhanced the expression of these mRNAs (P < 0.05), compared with the DE group.

Effects of HLWDD on expression of total Akt and p-Akt

Total Akt and phosphorylation of Akt were detected by Western blotting analyses. The images and histograms (Fig. 6C) illustrates that the treatments of metformin and HLWDD (3 and 6 g·kg⁻¹) had little impact on the expression of total Akt but obviously reversed the decreased ratio of p-Akt to Akt in the DE rats (P < 0.05).

Effect of HLWDD on expression of p-JNK, JNK, p-IRS-1 and IRS-1

P-JNK/JNK (Fig. 7A) and p-IRS-1 S307/IRS-1 (Fig. 7B) were detected for their close links with neuroinflammation. There were no significant differences in the ratios of JNK/β-actin or IRS-1/β-actin in the hippocampus of rats between the DE group and normal group. But the ratios of p-JNK/JNK and p-IRS-1 S 307/IRS-1 were markedly increased in the DE group, compared to the normal group (P < 0.01). The treatments of metformin and HLWDD (3 and 6 g·kg⁻¹) reduced the expressions of these protein (P < 0.05), compared with the DE group.

Quantitative HPLC analysis

Berberine is present as a main ingredient HLWDD with the highest content. With the method employed in the present study, berberine (Fig. 8C) was identified in HLWDD at 6.706 min by quantitative HPLC analysis (Fig. 8B). The content of berberine in HLWDD was 0.22% calculated through peak area ratio between its area in the test solution and standard solution, according to Chinese Pharmacopoeia (Fig. 8A).
Fig. 6 Effects of HLWDD on insulin signaling pathway in hippocampus. The relative expressions of InsR (A) and IRS-1 (B) mRNAs were detected in hippocampus, as well as the protein expression of p-Akt/Akt (C) (Mean expression of normal group = 1. Data were corrected with normal group and noted as Mean ± SEM, n = 3. *P < 0.05, **P < 0.01 vs Normal; *P < 0.05, **P < 0.01 vs DE)

Fig. 7 Effects of HLWDD on the expression of JNK, p-JNK, IRS-1 and p-IRS-1 in hippocampus. The relative expressions of JNK/β-actin, p-JNK/JNK (A), IRS-1/β-actin and p-IRS-1/IRS-1 (B) were measured and calculated by Image-J Launcher software. (Mean expression of normal group = 1. Data were corrected with normal group and noted as Mean ± SEM, n = 3. *P < 0.05, **P < 0.01 vs Normal; *P < 0.05, **P < 0.01 vs DE)
vulnerable to insulin resistance [24]. Therefore, the present study could ameliorate the increased blood glucose and prevent the rats from these harmful changes.

of rats with DE [25], were presented in cerebral coronary images in CA1 region of hippocampus were taken to show improvements in histopathological changes induced by diabetes after drug treatments. With the network of hyperglycemia in brain, the accumulation of amyloid protein and neuronal loss were increased in the DE rats, which as reversed by treatment with 3 and 6 g·kg⁻¹ of HLWDD, further confirming the neuroprotective activity of HLWDD in the DE rats by alleviating chronic hyperglycemia.

Recent studies have indicated insulin signaling pathway plays an important role in AD and diabetes [27]. The impairment of insulin signaling pathway may mediate the adverse changes in brain. After insulin binds to IR on the cell membrane, insulin signaling is delivered into cells and acts on IRS [28]. The abnormal changes in IRS induce abnormal regulations of proteins and enzymes, leading to damages in neurons in the end. In our study, the changes in insulin signaling pathway were also detected. The mRNAs expression of IR and IRS-1, the most important and best studied type of IRS, were decreased in hippocampus of the DE rats and the treatments of HLWDD could reverse the impairment of insulin signaling pathway by enhancing the expression of these genes.

Akt is one of the important kinases downstream of the insulin signaling pathway. Akt regulates not only the growth and development of neurons, but also learning and memory of the rats [29]. The activation of Akt also shows inhibiting effect on glycogen synthase kinase 3β [30], which causes Alzheimer’s pathology and neuronal loss in AD [31]. As a crucial kinase cascade with IRS-1, the activation of Akt was down-regulated when insulin signaling pathway was impaired, whereas the treatments of HLWDD alleviated this down-regulation in the DE rats. These data confirmed that the protective effects of HLWDD in brain might be based on its repairing of the dysfunctional network of insulin signaling pathway.

Although a series of physiological changes have been observed and reported in DE, there is no clear explanation for the pathogenesis yet. Chronic hyperglycemia also induces inflammatory response which damages memory and neurons [26]. Dating from the periphery induced by chronic hyperglycemia, pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1β, come cross the damaged blood brain barrier and intensified inflammatory response in brain [32]. In our study, proinflammatory cytokines in hippocampus were detected. The high levels of TNF-α, IL-6, and IL-1β indicated an increase in inflammatory response in hippocampus, whereas they were all decreased after treatments of 3 and 6 g·kg⁻¹ of HLWDD, suggesting an anti-inflammatory response in the brain of the DE rats.

The increasing inflammatory response would be a trigger of insulin resistance in brain. Therefore, we detected the related proteins on insulin signaling pathway. Recent studies have found factors, like proinflammatory cytokines, trigger the phosphorylation of IRS-1 at serine 307 site, which eliminates the physiological tyrosine phosphorylation of IRS-1 by activation of JNK [17]. In accordance with our results, HLWDD was found to have anti-inflammatory activity by inhibiting the release of inflammatory cytokines. Then, the expression of p-JNK and IRS-1 S307 were checked and found to be inhibited by administration of HLWDD, suggesting that

**Fig. 8** Quantitative HPLC analysis of HLWDD (A), berberine, a standard solution (B) and the structure of berberine (C)

**Discussion**

Hippocampus is important to learning and memory and vulnerable to insulin resistance [24]. Therefore, the present study was focused on hippocampus. In the present study, 50-day HGF diet combined with one STZ injection successfully induced hyperglycemia and resulted in the cognitive deficiency, neuronal damages, inflammatory response, and impaired insulin signaling pathway in the brain of the SD rats. The treatments with HLWDD (3 and 6 g·kg⁻¹) could ameliorate the increased blood glucose and prevent the rats from these harmful changes.

Abnormal hyperglycemia resulting from diabetes is regarded as a risk factor in the development of DE [2]. Therefore, FBG, TG, TC, and body weight were recorded in the present study. HLWDD treatment effectively controlled hyperglycemia in the DE rats.

Decreasing learning and memory capacity is a major behavior of deficient cognition. Morris water maze is an acknowledged method to assess the learning and memory ability since it was put forward in 1981. The significant improved cognitive function of the rats detected in Morris water maze test indicated the therapeutic effects of 3 and 6 g·kg⁻¹ of HLWDD in the DE rats.

On the other hand, amyloid deposition and neuronal loss, two significant Alzheimer’s pathological changes in the brain of rats with DE [25], were presented in cerebral coronary sections. CA1 region is considered as a most vulnerable region of hippocampus related to memory [24]. Therefore, images in CA1 region of hippocampus were taken to show improvements in histopathological changes induced by diabetes after drug treatments. With the network of hyperglycemia in brain, the accumulation of amyloid protein and neuronal loss were increased in the DE rats, which as...
the inflammatory response induced by hyperglycemia may be partly responsible for the impairment of insulin signaling pathway and the treatments of HLWDD repaired insulin signaling pathway by its inhibition on inflammatory response in hippocampus.

Based on traditional exploration and clinical experiences of Wendan decoction for almost 1000 years, HLWDD has been recorded on “Liu-yin-tiao-bian” since Qing dynasty. The doses of HLWDD used in our study were selected and calculated according to the primary dose of “Liu-yin-tiao-bian” used in the clinic. As a traditional prescription consisted of eight medicinal materials, the mechanism of action may be complex and mysterious to a certain degree. Previous studies have indicated that the treatment of HLWDD not only suppresses insulin resistance and inflammation in periphery of rats, but also alleviates cognitive deficits, decreasing release of Ach and oxidative damages in brain of patients with cognitive impairment [33]. In the present study, we tried to explore the potential efficacy of HLWDD in the DE rats and explored its mechanism on regulation of inflammation and insulin resistance in hippocampus. The results from the present study confirmed the cerebral protective effects of HLWDD and suggested that the mechanism of improvements in hippocampus of DE rats were related with its inhibition on the release of proinflammatory cytokines, which may induce inflammatory response in brain.

Metformin, a common anti-diabetic drug which shows protective activity in brain [34-35] as well as anti-inflammation effects [36], was used in the present study for comparison with HLWDD. Recent studies show metformin ameliorates neuronal insulin resistance and Alzheimer’s-like changes in mouse neuroblastoma cell line [37]. Metformin’s effects on promotion of spatial memory formation and neurogenesis are reported in adult mouse brain [38]. In our study, the improvement of metformin on memory and insulin signaling pathway were also observed. However, application of metformin is limited due to its side reactions. Metformin is difficult to be metabolized in liver, but easy to be excreted through kidneys. Thus, HLWDD may be a better agent considering its protective effects and hypotoxicity as a traditional Chinese herbal prescription, but future studies should explore the toxicity and metabolism of HLWDD in vivo.

Conclusion

In conclusion, we found that the treatment of HLWDD may alleviate the abnormal hyperglycemia-induced cognitive deficits, neuronal damages, and impaired insulin signaling pathway in hippocampus of the HGF + STZ-induced DE rats. HLWDD’s inhibition on release of pro-inflammatory cytokines and repair of insulin signaling pathway of HLWDD may underlie the mechanisms of its cerebral protective effects.

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