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Zuogui Jiangtang Jieyu Formula ameliorating hippocampal neuronal apoptosis in diabetic rats with depression by inhibiting JNK signaling pathway

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ABSTRACT

Objective To investigate the effect of Zuogui Jiangtang Jieyu Formula (左归降糖解郁方, ZJJF) on hippocampal neuron apoptosis in diabetic rats with depression and to ascertain whether its mechanism involves the regulation of JNK signaling pathway.

Methods (i) A total of 72 specific pathogen-free (SPF) grade male Sprague Dawley (SD) rats were randomly divided into six groups, with 12 rats in each group: control, model, metformin (Met, 0.18 g/kg) + fluoxetine (Flu, 1.8 mg/kg), and the high-, medium-, and low-ZJJF dosages (ZJJF-H, 20.52 g/kg; ZJJF-M, 10.26 g/kg; ZJJF-L, 5.13 g/kg) groups. All groups except control group were injected once via the tail vein with streptozotocin (STZ, 38 mg/kg) combined with 28 d of chronic unpredictable mild stress (CUMS) to establish diabetic rat models with depression. During the CUMS modeling period, treatments were administered via gavage, with control and model groups receiving an equivalent volume of distilled water for 28 d. The efficacy of ZJJF in reducing blood sugar and alleviating depression was evaluated by measuring fasting blood glucose, insulin, and glycated hemoglobin levels, along with behavioral assessments, including the open field test (OFT), forced swim test (FST), and sucrose preference test (SPT). Hippocampal tissue damage and neuronal apoptosis were evaluated using hematoxylin-eosin (HE) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. Apoptosis-related proteins Bax, Bcl-2, caspase-3, and the expression levels of JNK/Elk-1/c-fos signaling pathway were detected using Western blot and real-time quantitative polymerase chain reaction (RT-qPCR). (ii) To further elucidate the role of JNK signaling pathway in hippocampal neuronal apoptosis and the pharmacological effects of ZJJF, an additional 50 SPF grade male SD rats were randomly divided into five groups, with 10 rats in each group: control, model, SP600125 (SP6, a JNK antagonist, 10 mg/kg), ZJJF (20.52 g/kg), and ZJJF (20.52 g/kg) + Anisomycin (Aniso, a JNK agonist, 15 mg/kg) groups. Except for control group, all groups were established as diabetic rat models with depression, and treatments were administered via gavage for ZJJF and intraperitoneal injection for SP6 and Aniso for 28 d during the CUMS modeling period. Behavioral changes in rats were evaluated through the OFT, FST, and SPT, and hippocampal neuron damage and apoptosis were observed using HE staining, Nissl staining, TUNEL staining, and transmission electron microscopy (TEM). Changes in apoptosis-related proteins and JNK signaling pathway in the hippocampal tissues of rats were also analyzed.

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Results (i) ZJJF significantly reduced the high blood glucose, insulin, and glycated hemoglobin levels in model rats ($P < 0.01$). It increased autonomous activity and decreased despair-like behaviors ($P < 0.01$), improved the pathological damage of hippocampal neurons, increased the number of neuronal nuclei ($P < 0.01$), and reduced the number of mechanocytes, vacuolar cells, and apoptotic neurons ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively). ZJJF down-regulated the expression levels of pro-apoptotic proteins Bax and caspase-3 ($P < 0.01$), up-regulated the anti-apoptotic protein Bcl-2 ($P < 0.01$), and significantly inhibited the overexpression of phosphorylated JNK (p-JNK), Elk-1, and c-fos ($P < 0.01$). (ii) SP6 increased autonomous activity and reduced despair time in model rats ($P < 0.05$), although it had no significant effects on sucrose preference ($P > 0.05$). It increased the number of Nissl bodies in hippocampal neurons ($P < 0.01$), reduced the protein expression levels of Bax ($P < 0.01$) and caspase-3 ($P < 0.05$), and decreased the number of apoptotic neurons ($P < 0.05$). SP6 also increased the expression level of Bcl-2 ($P < 0.01$), and inhibited the high expression levels of p-JNK, Elk-1, and c-fos ($P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively), suggesting that hippocampal neuronal apoptosis in diabetic rats with depression is associated with abnormal activation of JNK signaling pathway. Compared with ZJJF group, ZJJF + Aniso group showed a decrease in sucrose preference ($P < 0.05$) and an increase in despair time ($P < 0.01$) with more notable hippocampal neuronal damage. This group also exhibited a decrease in expression level ($P < 0.01$) Bcl-2 and an increase in expression levels of Bax, caspase-3, p-JNK, Elk-1, and c-fos ($P < 0.01$, $P < 0.05$, $P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively), indicating that the antidepressant effects of ZJJF, its improvement of neuronal apoptosis, and regulation of JNK signaling molecules could all be reversed by a specific JNK agonist.

Conclusion ZJJF exerts a significant hypoglycemic effect and ameliorates the apoptosis of hippocampal neurons by inhibiting the activation of JNK signaling pathway, which is a promising formula for the treatment of diabetic depression in clinical settings.

1 Introduction

Diabetes mellitus (DM), a common metabolic disease, is characterized by persistent hyperglycemia. Its prevalence is anticipated to increase to 10.2% (578 million) of the global population by 2030 [1]. Prolonged hyperglycemia can lead to various complications, including retinopathy, diabetic nephropathy, and the increasingly recognized condition of diabetes-induced depression. In China, the incidence of depression among diabetic patients is reported to be 25.9% [2]. Clinical study has shown that people with diabetes have a two- to three-fold higher risk of experiencing depression compared to those without [3]. The coexistence of diabetes and depression can lead to a vicious cycle, intensifying each condition and significantly increasing the risks of self-harm and suicide among patients [4]. Furthermore, diabetic patients suffering from depression are more prone to develop macrovascular problems, making treatment more challenging and less effective [5]. Consequently, developing therapies that are capable of treating both diabetes and depression holds significant clinical importance.

In diabetic patients, persistent hyperglycemia and insulin resistance lead to microvascular dysfunctions in the brain, manifested as reduced cerebral blood flow [6]. Neuroimaging studies have shown that the incidence of white matter lesions and atrophy is relatively high in the hippocampus and amygdala of diabetic patients [7]. The hippocampus, which is crucial for learning and memory storage, plays a key role in the pathology of depression [8]. It is believed that neuronal apoptosis in the hippocampus is closely associated with the development of depression [9]. Research indicates that the hippocampus is particularly susceptible to prolonged hyperglycemic conditions, causing reduced neuronal activity and apoptosis, especially in its ventral region [10].

JNK, a key component of the mammalian mitogen-activated protein kinase (MAPK) superfamily, plays a crucial role in mediating neuronal apoptosis [11]. This subclass of serine/threonine protein kinases is activated by various extracellular stimuli, leading to the phosphorylation of transcriptional activators such as Elk-1. As a transcriptional activator, Elk-1 is activated through phosphorylation at the JNK Thr183 site. This activation influences the expression of the early gene c-fos and ultimately leads to hippocampal neuronal apoptosis [12, 13]. Study in diabetic mice has demonstrated that increased JNK phosphorylation correlates with enhanced neuronal apoptosis, suggesting that inhibition of the JNK pathway could mitigate stress-induced neuronal damage [14]. Importantly, JNK activation influences the balance of the Bcl-2 protein family, elevating pro-apoptotic proteins while reducing anti-apoptotic proteins, thereby initiating the mitochondrial apoptosis pathway in neurons, with caspase-3 serving as a critical executor [15, 16]. However, it remains unknown whether the alterations in associated apoptotic proteins in the hippocampus of diabetic depression are mediated through the JNK signaling pathway.

Traditional Chinese medicine (TCM) approaches

disease treatment holistically, engaging multiple pathways, channels, and targets to systematically regulate the body. This methodology offers unique advantages in managing complications. Zuogui Jiangtang Jieyu Formula (左归降糖解郁方, ZJJF) consists of Huangqi (Astragali Radix) 18 g, Shudihuang (Rehmanniae Radix Praeparata) 15 g, Guanyelianqiao (Forsythiae Fructus) 3 g, Shanzhuyu (Corni Fructus) 12 g, Danshen (Salviae Miltiorrhizae Radix et Rhizoma) 12 g, Gouqizi (Lycii Fructus) 12 g, Jianghuang (Curcumae Longae Rhizoma) 9 g, Tusizi (Cuscutae Semen) 9 g, Duzhong (Eucommiae Cortex) 9 g, Huainiuxi (Achyranthis Bidentatae Radix) 9 g, and Mudanpi (Moutan Cortex) 6 g. This herbal prescription is based on the famous traditional formula Zuogui Pill (左归丸), first recorded in the *Jing Yue Quan Shu* (《景岳全书》) from the Ming Dynasty, and commonly used in hypoglycemic therapy [17]. Previous clinical studies have demonstrated that ZJJF could effectively stabilize blood glucose levels, reduce blood viscosity, and alleviate depression, which could also protect hippocampal neurons from damage caused by excessive glucose, glutamate, and corticosterone in diabetic rats with depression [18, 19]. This study aims to clarify that the antidepressant and hypoglycemic effects of ZJJF are related to the inhibition of hippocampal neuronal apoptosis by regulating JNK signaling pathway.

2 Materials and methods

2.1 Drugs and quality control

All the medicinal materials for ZJJF were sourced from The First Hospital of Hunan University of Chinese Medicine (Changsha, China) and were authenticated by Professor WANG Yuhong. To ensure the quality and thereby warrant the effectiveness, the chromatographic fingerprint of ZJJF was established and characterized using high-performance liquid chromatography (HPLC). Chromatographic separation was achieved using an ACQUITY UPLC® C18 column, with a mobile phase consisting of A (water) and B (methanol). The gradient elution conditions were as follows: 0 – 5 min, 25% B; 5 – 15 min, 35% B; 15 – 25 min, 42% B; 25 – 50 min, 45% B; 50 – 60 min, 80% B; 60 – 75 min, 90% B. Operating conditions were as follows: detection wavelength, 354 nm; column temperature, 35 °C; flow rate, 1.0 mL/min; injection volume, 10 μL. The qualitative analysis was carried out by HPLC with the standards of verbascoside, hyperoside, paeonol, cryptotanshinone, and tanshinone IIA, which were the most representative and easily detectable major components in the ZJJF extracts. Metformin (Met) was purchased from Xiangya Pharmaceutical Company, China, and fluoxetine (Flu) was obtained from Patheon, France.

2.2 Reagents

Streptozotocin (STZ, Sigma-Aldrich, USA), SP600125 (SP6), Anisomycin (Aniso) (MedChemExpress, USA), insulin and HbA1c enzymatic kits (Elabscience, China), radioimmunoprecipitation assay (RIPA) buffer, protease inhibitor, hematoxylin and eosin (HE) staining kit, Nissl staining kit, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining kit (Servicebio, China), phosphorylated JNK (p-JNK) and JNK antibody (Abcam, USA), primary antibodies against Elk-1, c-fos, Bcl-2, Bax, and cleaved caspase-3 (Proteintech, China), horseradish peroxidase-conjugated secondary antibody (Boster, China), reverse transcription kit and SYBR Green (TaKaRa, Japan), TRIzol (Invitrogen, USA), and primers (Sangon Biotech, China).

2.3 Instruments

HPLC system (Waters, Arc Premier), blood glucose meter (Sinocare, GA-3), animal behavior analysis system (Xinruan, VisuTrack), inverted biological microscope (Carl Zeiss, Primovert), tissue embedding machine (Leica, Arcadia H), paraffin microtome (Leica, RM2235), transmission electron microscope (Hitachi, HT7800), digital microtome scanning analysis system (3DHISTECH, Pannoramic MIDI), microspectrophotometer (Thermo Fisher, NanoDrop), polymerase chain reaction (PCR) amplification apparatus (Illumina, ECO), and chemiluminescence imaging system (Bio-Rad, ChemiDoc MP).

2.4 Experimental animals

In this study, a total of 130 male Sprague Dawley (SD) rats [specific pathogen-free (SPF) grade, 200 – 220 g, approximately seven weeks old] were used. All rats were purchased from Hunan Slake Jingda Experimental Animal Co., Ltd. [Changsha, China; laboratory animal quality certificate No. 430727211101417753; laboratory facilities certificate No. SCXK (Xiang) 2019-0004]. All relevant regulations and principles were strictly followed during all experimental procedures. The study was also approved by the Experimental Animal Ethics Committee of Hunan University of Chinese Medicine (LLBH-202012130001).

2.5 Grouping, modeling, and drug administration

A total of 80 rats were randomly divided into two groups, with 12 rats in control group and the remaining 68 rats in treatment group, receiving a high-fat diet combined with STZ (38 mg/kg, i.v.) for diabetes modeling. First, the 68 rats received a high-fat diet by gavage for 14 d. Then, freshly prepared STZ dissolved in citrate buffer was injected into the tail vein after overnight fasting. To ensure the success of diabetic rat modeling, fasting blood glucose levels were measured 72 h after treatment, with a level of no less than 16.7 mmol/L indicating successful diabetic modeling. Rats that did not survive the modeling process were excluded from the study. Finally, 60 diabetic rats successfully modeled were selected and randomly divided into model, Met (0.18 g/kg) + Flu (1.8 mg/kg), and

high-, medium-, and low-ZJJF dosages (ZJJF-H, ZJJF-M, and ZJJF-L) groups, with 12 rats in each group. The dosages for ZJJF, calculated based on clinical equivalent dosages, were 20.52, 10.26, and 5.13 g/kg, respectively. These diabetic rat models subsequently underwent chronic unpredictable mild stress (CUMS) treatment for 28 d to induce depression [18-20]. The rats were administered a drug volume of 10 mL/kg via oral gavage, starting from the first day of CUMS modeling and continuing for 28 consecutive days.

To further elucidate the role of JNK signaling pathway in hippocampal neuronal apoptosis in diabetic rats with depression and to determine if ZJJF mediates its therapeutic effects by targeting JNK, we selected additional 50 rats and randomly divided into five groups: control, model, SP6 (a JNK antagonist), ZJJF (20.52 g/kg), and ZJJF (20.52 g/kg) + Aniso (a JNK agonist) groups, with 10 rats in each group. All procedures replicating those previously described. SP6 and Aniso were administered intraperitoneally from the first day of CUMS modeling for 28 consecutive days, and the doses were 10 and 15 mg/kg daily, respectively [21, 22].

2.6 Behavioral assessments

After the administration period, behavioral tests were conducted to identify depressive-like behaviors in the rats in a light/dark box and a quiet environment. Two impartial observers who were blinded to the treatment protocols, scored the behaviors.

2.6.1 Open-field test (OFT) The OFT assessed spontaneous locomotor activities in rats using an 80 cm × 80 cm × 40 cm apparatus uniformly divided into 25 squares to facilitate precise movement measurement. To acclimate the rats to the experimental settings, they were placed in the test room in darkness for 1 h prior to testing. During the test, each rat was placed in the center of the apparatus. Horizontal movements were quantified by counting the number of squares crossed by the rat, and vertical movements were measured by recording the number of instances when the rat raised both front paws. Both types of movements were recorded over a period of 4 min.

2.6.2 Forced swim test (FST) The FST was conducted to observe behavioral changes in rats under conditions of despair, using a cylindrical plexiglass swimming tank with a height of 40 cm, a diameter of 20 cm, and a water depth of approximately 30 cm; the water temperature was maintained at 24 ± 1 °C. During the test, acute forced swim stress was applied to each rat for 5 min, with immobile time (the duration when a rat ceased all active movements and floated passively) being recorded by observers.

2.6.3 Sucrose preference test (SPT) The SPT aimed to assess anhedonia, a key depressive symptom, in rats. Initially, rats underwent a training period where they had access to two bottles containing 1% sucrose solution for 24 h; subsequently, one bottle was replaced with water for another 24 h. After training, rats were deprived of water for 12 h but not fasted. Subsequently, they were given unlimited access to 1% sucrose solution in one bottle and water in the other for 4 h, with the positions of the bottles switched every 2 h to prevent location bias. The sucrose preference rate = volume of sucrose solution consumed/(volume of sucrose solution consumed + volume of water consumed) × 100 %.

2.7 Blood glucose, insulin, and HbA1c analysis and sampling

Blood samples were collected from the rats' tail veins to measure glucose levels using a glucometer, following a 12 h fasting period. This procedure was carried out weekly. Upon completion of the final glucose measurement, all animals were anesthetized with pentobarbital sodium at a dose of 60 mg/kg, and then euthanized. Blood was subsequently collected from the abdominal aorta, centrifuged to separate serum, and analyzed for fasting insulin and HbA1c levels using enzymatic assay kits. Immediately after blood collection, the brains were extracted, and the hippocampus was carefully dissected on ice, then preserved at – 80 °C for subsequent analyses. Additionally, for morphological studies, the entire brain was perfused through the ventricles and then fixed in 4% paraformaldehyde.

2.8 Histopathological staining

Brain tissues fixed in 4% paraformaldehyde solution underwent a series of preparatory steps for histological examination. Following fixation, the tissues were sequentially dehydrated in graded ethanol solutions before being cleared in xylene to ensure full transparency. The tissues were then embedded in paraffin and sectioned into 5 μm thick slices using a microtome. For staining, sections underwent HE, Nissl, and TUNEL staining according to manufacturers' protocols. After staining, sections were dehydrated again in an ascending ethanol series, cleared once more in xylene, and mounted with neutral resin to preserve the stains' integrity. Quantitative analysis of the stained sections was performed using ImageJ software, focusing on metrics such as cell count and the area of positive staining.

2.9 Observation by transmission electron microscopy (TEM)

A fragment of hippocampal tissue (approximately 1 mm^3) was carefully excised and immediately immersed in electron microscope fixative. The tissue was fixed in this solution at 4 °C for 24 h to ensure optimal preservation of ultrastructural details. After fixation, the tissue was dehydrated through a graded series of ethanol, was embedded in

epoxy resin, and then sectioned into ultrathin slices of 50 nm thickness using an ultramicrotome. These slices were stained with uranyl acetate followed by lead citrate, mounted on copper grids, and examined under TEM to observe ultrastructural changes in hippocampal neurons.

2.10 Western blot analysis

The hippocampal tissue was homogenized in RIPA buffer with protease inhibitors. Next, the supernatant was extracted after centrifugation and stored at – 20 °C. Subsequently, the protein concentration of the supernatant was measured using a microspectrophotometer. After measurement, the gel was prepared according to the molecular weight of the target protein, and 30 μg of the protein sample was added to the sample buffer and denatured by boiling. Subsequently, protein separation was achieved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% skimmed milk at room temperature for 2 h, and then incubated overnight at 4 °C in primary antibodies against p-JNK (1 : 2 000), JNK (1 : 5 000), Elk-1 (1 : 2 000), c-fos (1 : 2 000), Bax (1 : 2 000), Bcl-2 (1 : 2 000), and cleaved caspase-3 (1 : 2 000), respectively. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1 : 5 000) for 2 h at room temperature. The bands on the membrane were visualized using an enhanced chemiluminescence detection system, and the average gray value of each band was measured using Quantity One software V4.6.2.

2.11 Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from 100 mg of hippocampal tissue using TRIzol reagent. RNA quality and concentration were measured using a NanoDrop spectrophotometer, showing a concentration of 200 ng/μL and an A260/A280 ratio of 2.0, indicating high purity. This RNA was then reverse transcribed into cDNA, which was used for RT-qPCR amplification under the following conditions: predenaturation at 95 °C for 15 min, denaturation at 95 °C for 10 s, annealing and extension at 60 °C for 30 s, for a total of 40 cycles. The Cq values of the PCR products of the target gene and internal reference gene (*β*-actin) were determined, and the relative expression levels of mRNA of each target gene were analyzed by the $2^{-\Delta\Delta CT}$ method. The primer sequences are summarized in Table 1.

Table 1 Primer sequences for the RT-qPCR analysis

Gene	Forward primer (5′ – 3′)	Reverse primer (5′ – 3′)	Amplicon length (bp)
JNK	AGGAATAGTGTGTGCAGCTTATG	CTTCTAGGGATTTCTGTGGTGTG	188
Elk-1	TGCTCCACTGAAGACTGCC	GGCTGCAGGGACTGTATTG	224
C-fos	GAGCCGGTCAAGAACATTAGC	GAAGACGTATAGGTAGTGCAGCTG	260
Bcl-2	TGTGTGGAGAGCGTCAACAG	TCCACAAAGGCGTCCCAGC	128
Bax	GTTTCATCCAGGATCGAGC	GATCATCCTCTGCAGCTCC	155
Caspase-3	GCAAGACAACTCGAGCCTG	CATTCTTCTCTCTGTGCTTGATCG	163
β-Actin	CATCCTGCGTCTGGACCTGG	TAATGTCACGCACGATTTCC	116

2.12 Statistical analysis

Data were evaluated using one-way analysis of variance (ANOVA) in SPSS, followed by Fisher's least significant difference (LSD) post hoc test. All values were reported as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

3 Results

3.1 Characteristics of the HPLC fingerprints of ZJJF

As shown in Figure 1, the retention times of the five components were 13.87, 17.85, 31.54, 56.37,and 59.03 min, respectively, and the sample peaks corresponded to the standards. Based on the peak areas of the mixed standards and sample solutions, the contents of the five components were calculated, with the content of verbascoside derived from Shudihuang (Rehmanniae Radix Praeparata) being the highest at 2.569 mg/g. The specific content of each component is shown in Table 2.

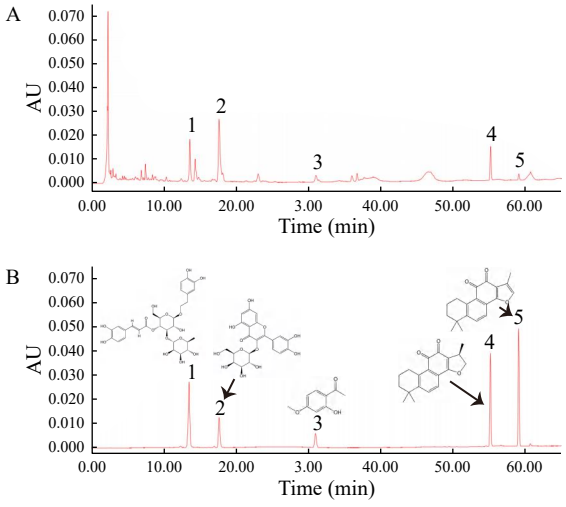

Figure 1 The HPLC fingerprints of the main components of ZJJF extracts

A, sample chromatogram map. B, mixed standard chromatogram map. AU, absorbance units. 1, verbascoside. 2, hyperoside. 3, paeonol. 4, cryptotanshinone. 5, tanshinone IIA.

Table 2 Quantitative analysis of the major components in ZJJF extracts

Standard	Component	Amount (mg/g)	Ascription
1	Verbascoside	2.5690	Shudihuang (Rehmanniae Radix Praeparata)
2	Hyperoside	0.5263	Guanyelianqiao (Hypericum perforatum)/Tusizi (Cuscutae Semen)
3	Paeonol	1.2630	Mudanpi (Moutan Cortex)
4	Cryptotanshinone	0.7684	Danshen (Salviae Miltiorrhizae Radix et Rhizoma)
5	Tanshinone IIA	0.8842	Danshen (Salviae Miltiorrhizae Radix et Rhizoma)

3.2 ZJJF improving hyperglycemia and depressive symptoms

Diabetes with comorbid depression has two hallmark symptoms from both conditions: hyperglycemia from diabetes and behavioral despair from depression. In our study, we simultaneously evaluated blood glucose status and depression-like behaviors in diabetic rats with depression. We observed significantly elevated fasting blood glucose (Figure 2A), insulin (Figure 2B), and HbA1c levels (Figure 2C) in rats in model group compared with control group ($P < 0.001$), along with decreased scores of locomotor activity in the OFT (Figure 2D, $P < 0.001$) and increased immobility time in the FST (Figure 2E, $P < 0.001$). Compared with model group, treatment with ZJJF-H or Met + Flu noticeably lowered blood glucose level, as well as insulin and HbA1c levels ($P < 0.001$), alleviating depressive symptoms in rats through enhanced activity ($P < 0.001$) and reduced despair-like behaviors ($P < 0.01$). Similarly, ZJJF-M effectively improved hyperglycemia and depressive symptoms in rats, demonstrating ZJJF's potent hypoglycemic and antidepressant properties.

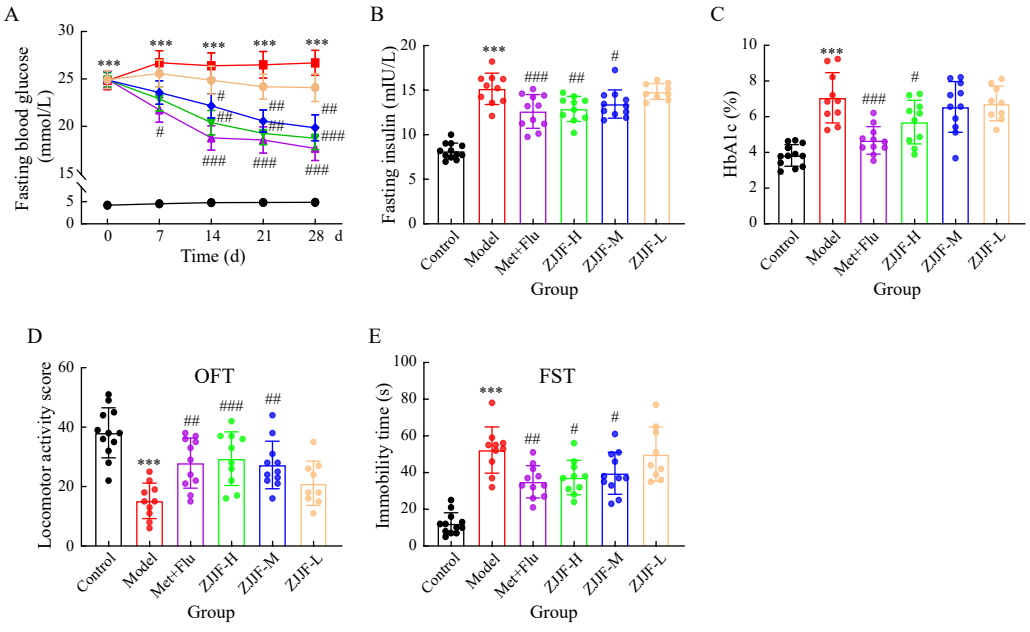

Figure 2 Effects of ZJJF on blood glucose, insulin, HbA1c, and behavioral performances in diabetic rats with depression

A, the fasting blood glucose level. B, the fasting insulin level. C, the HbA1c level. D, the locomotor activity score in OFT. E, the immobile time in FST. Data were represented as mean ± SD. $^{***}P < 0.001$, compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared with model group.

3.3 ZJJF alleviating hippocampal neuronal damages

The hippocampus serves as the brain's hub for learning, memory, and emotional-behavioral regulation, making it a critical focus for depression research and the most vulnerable part of the brain to hyperglycemia. In control group, hippocampal CA1 area neurons were arranged neatly and tightly, with normal intercellular space and intact morphological structure. In contrast, the neurons of rats in model group exhibited atrophy, swelling, vacuolation, scattered arrangement, and cell necrosis (Figure 3A). Quantitative analysis found that the number of cell nuclei (Figure 3B) of rats in model group was remarkably reduced ($P < 0.001$), and the number of mechanocytes (Figure 3C) and vacuolar cells (Figure 3D) increased ($P < 0.001$) compared with control group. Treatment of ZJJF-H significantly mitigated the adverse effects previously observed, significantly increased the number of nuclei in rats ($P < 0.01$), and decreased the number of mechanocytes and vacuolar cells ($P < 0.05$ and $P < 0.01$, respectively).

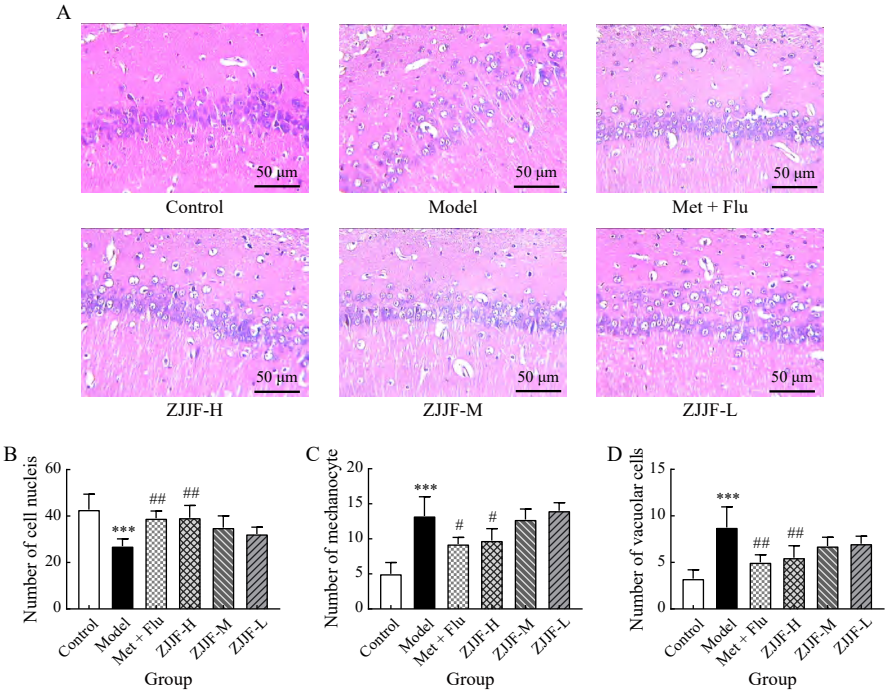

Figure 3 Effects of ZJJF on the pathological morphology of hippocampus in diabetic rats with depression by HE staining

A, neuronal morphology in the hippocampal representative CA1 area (× 200). B, the number of cell nucleis. C, the number of mechanocytes. D, the number of vacuolar cells. $^{***}P < 0.001$, compared with control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with model group.

3.4 ZJJF attenuating hippocampal neuronal apoptosis

TUNEL staining indicated a significant increase in apoptotic cells in the hippocampal CA1 area of diabetic rats with depression (Figure 4A and 4B, $P < 0.001$). Meanwhile, the protein expression levels of Bax and cleaved caspase-3 (Figure 4C – 4F) significantly increased ($P < 0.001$), while Bcl-2 decreased ($P < 0.001$). Treatment with ZJJF-H or Met + Flu significantly reduced the number of apoptotic neurons ($P < 0.01$), decreased expression levels of Bax and cleaved caspase-3 ($P < 0.01$), and increased expression level of Bcl-2 ($P < 0.001$). The ratio of Bax/Bcl-2 was significantly lower in all administration groups ($P < 0.001$). The mRNA expression levels of the target molecules also confirmed the results above (Figure 4H – 4J). These results suggest that ZJJF is effective in combating depression in diabetic rats, underscoring the necessity for further research to elucidate the specific pathways through which it regulates apoptosis-related proteins.

3.5 ZJJF inhibiting the JNK signaling pathway

JNK, a key signaling molecule related to cell apoptosis, controls the expression of downstream proteins Elk-1 and c-fos through autophosphorylation, ultimately leading to neuronal apoptosis [11]. In our study, the ratio of p-JNK/JNK was obviously up-regulated in model group (Figure 5A and 5B, $P < 0.001$), highlighting a state of heightened JNK activation. Subsequently, JNK activation also resulted in increased Elk-1 and c-fos protein expression levels (Figure 5A, 5C, and 5D, $P < 0.001$). In addition, these trends were confirmed by detecting the mRNA expression levels of the above proteins in the hippocampus (Figure 5E – 5G). Compared with model group, ZJJF-H significantly down-regulated the expression levels of Elk-1 and c-fos ($P < 0.01$).

3.6 Aniso reversing the antidepressant and neuroprotective effects of ZJJF

To further elucidate the role of JNK in hippocampal neuronal apoptosis in diabetic rats with depression, we employed a JNK antagonist (SP6) and investigated ZJJF's therapeutic effects under JNK agonism. Findings showed that SP6 significantly enhanced locomotor activity in the OFT and reduced immobility time in the FST compared with model group ($P < 0.05$), but did not affect sucrose preference (Figure 6A and 6B). Conversely, Aniso, an agonist of JNK, negated the beneficial effects of ZJJF in increasing the sucrose preference and decreasing immobility time ($P < 0.01$ and $P < 0.05$, respectively, Figure 6B and 6C). Histopathological staining and quantitative analysis confirmed that JNK inhibition had anti-apoptotic and neuroprotective effects by reducing hippocampal neuronal damage and apoptosis (Figure 6D – 6F). TEM results showed that the rat models had blurred and irregular neuronal nuclear membranes, with remarkable reduced cytoplasmic edema and vacuole enlargement (Figure 6G). After administration of ZJJF or SP6, neuronal

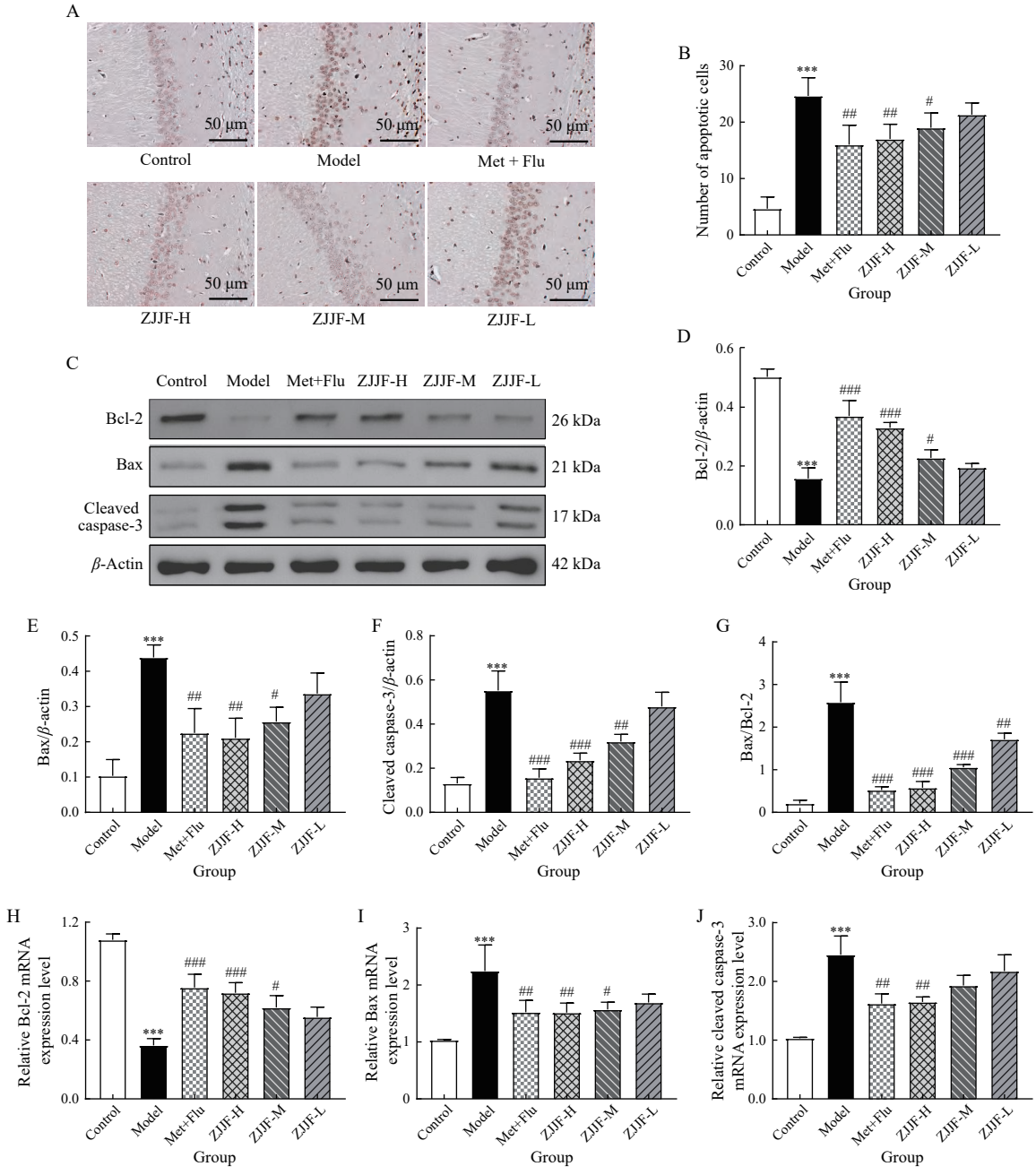

Figure 4 Effects of ZJJF on hippocampal neuronal apoptosis in diabetic rats with depression

A, TUNEL staining of neuronal apoptosis in the hippocampus representative CA1 region (× 200). B, the number of apoptotic cells in TUNEL staining analysis. C, Western blot representative protein bands of Bcl-2, Bax, and cleaved caspase-3 in hippocampus. D – G, relative expression levels of Bcl-2, Bax, cleaved caspase-3 proteins, and the ratio of Bax/Bcl-2, respectively. H – J, RT-qPCR analysis of Bcl-2, Bax, and cleaved caspase-3 mRNA expression levels in the hippocampus, respectively. $^{***}P < 0.001$, compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared with model group.

pathological damages in rats were significantly reduced when compared with model group, with reduced apoptotic cells in the hippocampal CA1 area ($P < 0.01$ and $P < 0.05$, respectively), and markedly increased Nissl bodies ($P < 0.01$). Interestingly, compared with ZJJF group alone, ZJJF+ Aniso significantly reversed sucrose preference ($P < 0.05$), immobility time ($P < 0.01$), and the number of Nissl bodies ($P < 0.05$), also increased neuronal structural damages, suggesting that Aniso could partially counteract ZJJF's effects.

3.7 Aniso in conjunction with ZJJF reversing the expression levels of the JNK signaling and apoptosis-related proteins

Western blot analysis was conducted to delineate the impact of JNK antagonists and agonists on hippocampal apoptosis-related proteins and the JNK signaling pathway in diabetic rats subjected to CUMS. Our results revealed that rats administered with SP6 effectively up-regulated anti-apoptotic Bcl-2 protein expression level (Figure 7A,

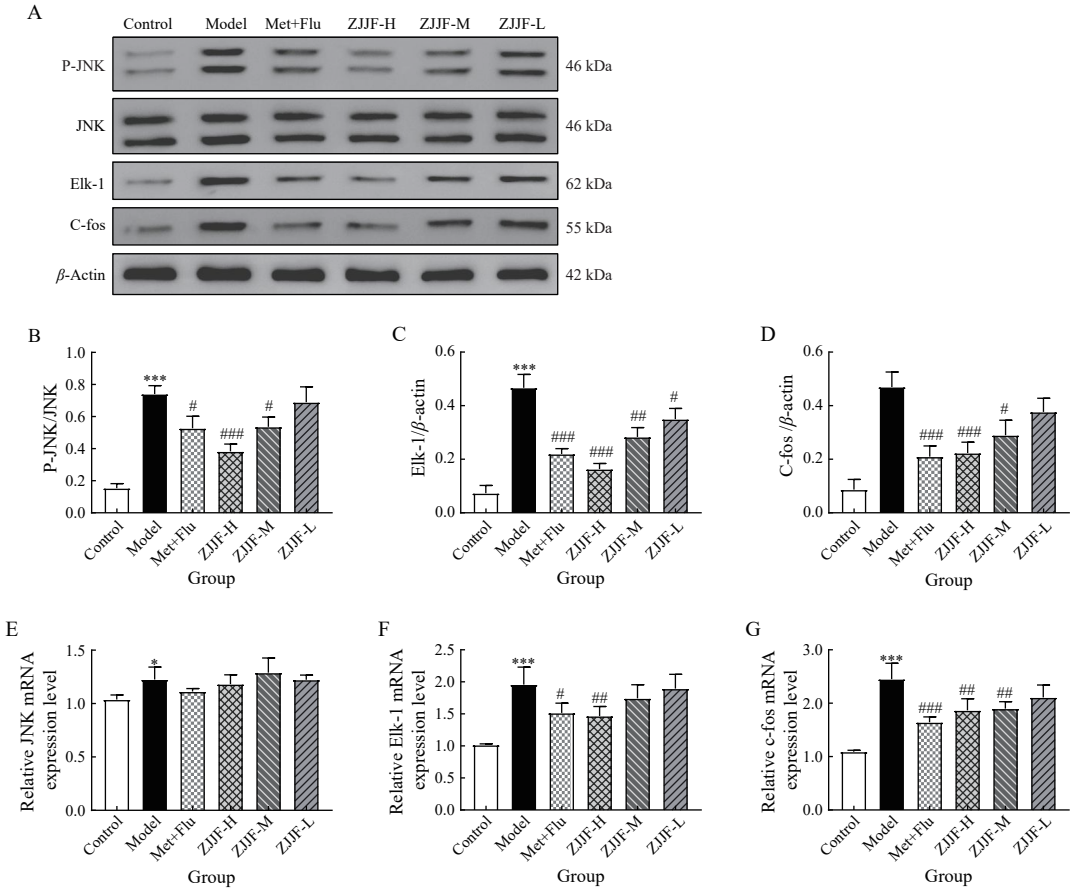

Figure 5 Effects of ZJJF on the expression levels of JNK/Elk-1/c-fos pathway in diabetic rats with depression

A, Western blot representative protein bands of p-JNK, JNK, Elk-1, and c-fos in the hippocampus. B – D, the ratio of p-JNK/JNK, relative expression levels of Elk-1 and c-fos proteins, respectively. E – G, RT-qPCR analysis of JNK, Elk-1, and c-fos mRNA in the hippocampus, respectively. $^{*}P < 0.05$ and $^{***}P < 0.001$, compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared with model group.

$P < 0.001$) and inhibited the expression levels of Bax (Figure 7B, $P < 0.001$), cleaved caspase-3 (Figure 7C, $P < 0.05$), p-JNK (Figure 7D, $P < 0.001$), Elk-1 (Figure 7E, $P < 0.001$), and c-fos (Figure 7F, $P < 0.05$). These results underscored JNK's critical role in driving hippocampal neuron apoptosis in diabetic rats with depression, and suggested that inhibiting the expression of JNK would exert a similar effect to that of therapeutic drugs. We also found that the regulatory effect of ZJJF on the aforementioned proteins in model rats could be blocked by Aniso. Interestingly, the protein expression levels were not statistically different from those in model group after Aniso reversed the above trends, except for Elk-1, which still demonstrated significantly low expression ($P < 0.05$). All these results indicated that the mechanism of ZJJF's efficacy is related to the inhibition of JNK signaling pathway and its mediated cellular apoptosis.

4 Discussion

Establishing stable and reliable animal models is essential for exploring disease pathologies and assessing pharmacodynamics. Previous study has demonstrated that combining STZ tail vein injections with CUMS effectively replicates diabetic rat models with depression, exhibiting hallmark features of both conditions [19]. In this study, rats exhibited significantly elevated blood glucose levels post-modeling, alongside aberrant behavioral patterns including limited autonomy, increased immobility time, and anhedonia, one of the core symptoms of depression. These findings confirmed the successful establishment of diabetic rat models with depression. Following ZJJF treatment, there was a notable reduction in blood glucose, insulin, and HbA1c levels in the rat models, along with a significant alleviation of depressive behaviors, whose efficacy could be confirmed by comparison with positive control drugs.

Patients with type 2 diabetes exhibiting more severe depression often face poorer glycemic control [23]. Study has shown that patients receiving antidepressant treatment have significantly better glycemic control over the long term compared with untreated patients [24]. ZJJF is based on the hypoglycemic prescription Zuogui Pill with the addition of Jianghuang (Curcumae Longae Rhizoma) and Guanyelianqiao (Forsythiae Fructus), both of which are widely recognized for their antidepressant and neuroprotective properties. Guanyelianqiao (Forsythiae

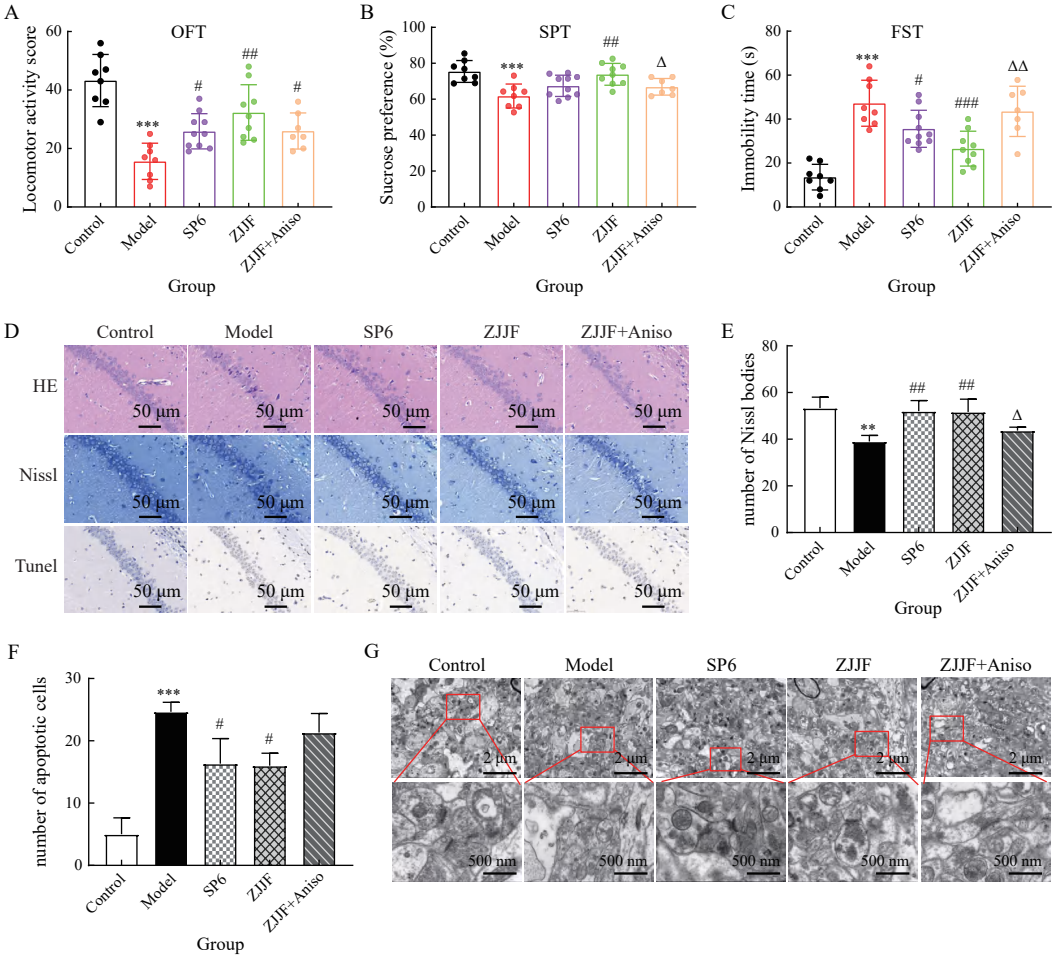

Figure 6 Effects of ZJJF + Aniso on behavioral performances and hippocampal morphology in diabetic rats with depression

A – C, behavioral tests of OFT, SPT, and FST, respectively. D, representative histopathologic staining of the hippocampal CA1 region (× 200). E, the number of Nissl bodies in Nissl staining analysis. F, the number of apoptotic cells in TUNEL staining analysis. G, ultrastructure of hippocampal neurons in TEM (× 5 000 and × 20 000). $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared with model group. $^{\Delta}P < 0.05$ and $^{\Delta\Delta}P < 0.01$, compared with ZJJF group.

Fructus) has been used as a first-line treatment for major depression in some European countries. The composition of ZJJF, including Huangqi (Astragali Radix), Shudihuang (Rehmanniae Radix Praeparata), Tusizi (Cuscutae Semen), Duzhong (Eucommiae Cortex), Danshen (Salviae Miltiorrhizae Radix et Rhizoma), and Mudanpi (Moutan Cortex), not only provides hypoglycemic benefits but also exhibits antidepressant effects. Specifically, verbascoside, mainly derived from Shudihuang (Rehmanniae Radix Praeparata), prevents neuronal apoptosis and exerts antidepressant effects [25]. Hypericin, a potential antidepressant compound, inhibits hippocampal neuronal apoptosis and autophagy to exert neuroprotective effects [26]. Paeonol from Mudanpi (Moutan Cortex) offers both hypoglycemic and neuroprotective effects against diabetic neuropathy [27]. Cryptotanshinone, a key extract from Danshen (Salviae Miltiorrhizae Radix et Rhizoma), attenuates depressive symptoms in CUMS rats by modulating the intestinal microbiota and the PI3K-AKT pathway [28]. Tanshinone IIA has also been reported to have neuroprotective effects and to ameliorate depressive-like behavior in rats [29]. These pivotal components underscore ZJJF's multifaceted therapeutic potential, with its quality and composition confirmed through HPLC analysis.

The hippocampus, central to emotion and cognition, often shows signs of atrophy, increased apoptosis, and neurotrophic deficits in patients with depression [30]. Diabetic rats frequently show cognitive dysfunction, which is associated with hippocampal neuronal damage [31]. After treatment with ZJJF, the number of hippocampal neurons in diabetic rats with depression normalized, and pathological damages such as swelling and deep staining were markedly reduced. Alterations in the JNK signaling are strongly associated with hippocampal neuronal damage [32]. Recent studies have emphasized that hippocampal damage caused by diabetes or depression is associated with the abnormal activation of the JNK signaling pathway, leading to abnormal expression levels of apoptosis-related proteins [33, 34].

The role of JNK activation in inducing neuronal apoptosis through transcriptional regulation of apoptotic

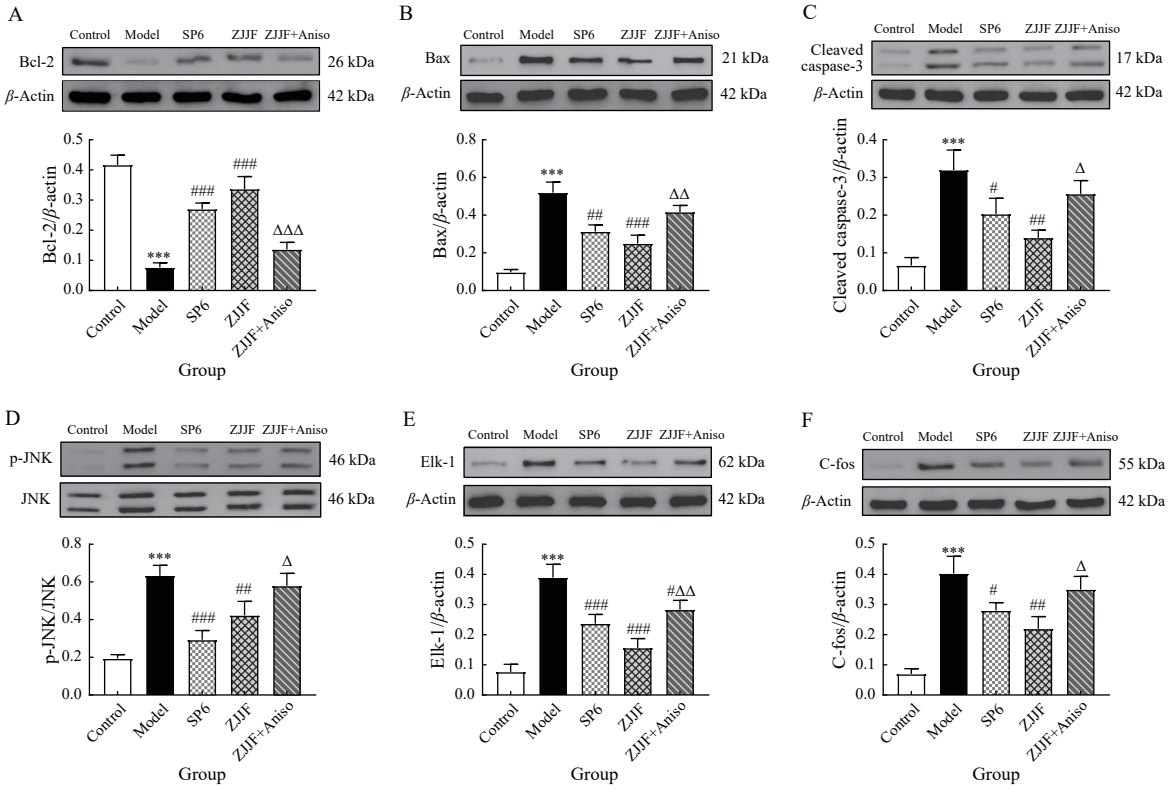

Figure 7 Effects of ZJJF + Aniso on the expression levels of apoptosis related protein and JNK signaling pathway in the hippocampus of diabetic rats with depression

A – F, Western blot analysis of Bcl-2, Bax, cleaved caspase-3, p-JNK, Elk-1, and c-fos in hippocampus, respectively. $^{***}P < 0.001$ compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared with model group. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, and $^{\Delta\Delta\Delta}P < 0.001$, compared with ZJJF group.

proteins (e.g., Bim, Bax, and Bcl-2) is well-established [35]. Elk-1, a transcriptional activator and one of the nuclear substrates of JNK, is involved in cell differentiation, proliferation, and apoptosis. It triggers neuronal apoptosis via c-fos regulation, a key marker of neurological activity [36]. While basal c-fos levels are stable in the central nervous system under normal circumstances, its expression increases significantly in response to exogenous stimulation signal in neurons, playing an important role in mediating neuronal apoptosis [37]. However, whether JNK and its regulated Elk-1/c-fos signaling are involved in the occurrence of diabetic depression has not yet been reported. In our study, we found that diabetic rats with depression had considerably higher expressions levels of p-JNK, Elk-1, and c-fos in the hippocampus when compared with normal rats, whereas their levels were significantly reduced when encountered with specific JNK antagonists. These results suggest that abnormal activation of JNK plays a key role in mediating the apoptosis of hippocampal neurons in diabetic depression.

Furthermore, the JNK signaling pathway regulates the activity of the Bcl-2 family members and key regulators of apoptosis through the mitochondrial pathway [38]. The Bcl-2 family, including Bcl-2 and Bax, comprises genes closely related to cellular apoptosis. Bax forms homodimers to promote cellular apoptosis, while Bcl-2 forms heterodimers to inhibit apoptosis. Therefore, the relative balance between the two determines the fate of cells [39]. It was found that an increased Bax/Bcl-2 ratio in the hippocampus of diabetic rats led to neuronal apoptosis, which was more significant in depressed rats subjected to CUMS [40, 41]. Moreover, the Bcl-2 family mediates apoptosis based on mitochondrial integrity by activating caspases, a class of apoptosis-executing proteins. Caspase-3, a crucial caspase subtype, acts as a primary facilitator of the apoptotic pathway and can initiate apoptosis following oligomerization [42]. Our study found that ZJJF inhibited the phosphorylation of JNK, decreased the expression of Elk-1, c-fos, Bax, and cleaved caspase-3, and reduced the number of apoptotic neurons. Simultaneously, ZJJF increased the expression of the anti-apoptotic protein Bcl-2, thereby mitigating hippocampal neuron damage and offering both hypoglycemic and antidepressant benefits. Notably, the therapeutic effects of ZJJF on these molecular pathways were largely nullified when co-administered with Aniso, a JNK agonist, suggesting the crucial role of JNK modulation in ZJJF's efficacy.

To sum up, this study highlights the pivotal role of JNK overactivation in the apoptosis of hippocampal neurons in diabetic rats with depression. Crucially, treatment with ZJJF ameliorates neuronal apoptosis by inhibiting the hippocampal JNK/Elk-1/c-fos signaling pathway (Figure 8). This finding positions ZJJF as a promising

therapeutic agent for diabetic depression, underscoring its potential to counteract the detrimental effects of the JNK pathway dysregulation on neuronal function. However, this study alone is not sufficient to fully explain the efficacy of ZJJF, and we will continue to conduct a further studies to further support this promising drug and provide a new solution for the clinical treatment of diabetic depression.

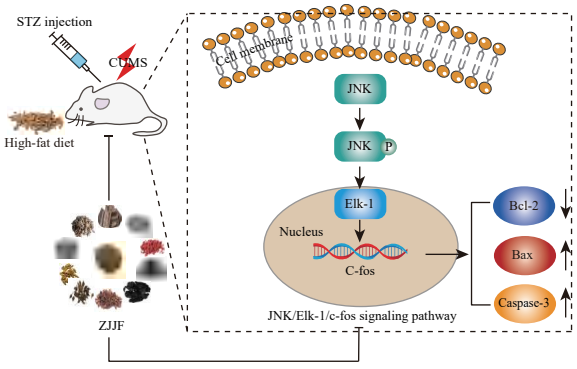

Figure 8 ZJJF exerts hypoglycemic and antidepressant effects by inhibiting the JNK/Elk-1/c-fos signaling pathway
P represents phosphorylation.

5 Conclusion

ZJJF effectively attenuates hippocampal neuronal apoptosis by inhibiting the JNK/Elk-1/c-fos signaling pathway. This beneficial effect is reversed by a specific JNK agonist, indicating the crucial role of JNK signaling in the therapeutic action of ZJJF. Additionally, ZJJF's favorable effects on lowering blood glucose levels and alleviating depressive-like behaviors are linked to its regulation of JNK signaling. These findings highlight the potential of ZJJF as a dual-purpose treatment for managing both diabetic and depressive conditions, offering insights into its mechanisms of action and therapeutic potential.

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Competing interests

The authors declare no conflict of interest.

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左归降糖解郁方通过抑制 JNK 信号通路改善糖尿病并发抑郁症大鼠海马神经元凋亡

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【摘要】目的 探讨左归降糖解郁方（ZJJF）对糖尿病并发抑郁症大鼠海马神经元凋亡的影响，并明确其作用机制是否与调控 JNK 信号通路有关。**方法** （1）将 72 只无特定病原体（SPF）级雄性 Sprague Dawley（SD）大鼠随机分为 6 组，每组 12 只：对照组、模型组、二甲双胍（0.18 g/kg）+氟西汀（1.8 mg/kg）组和 ZJJF 高（20.52 g/kg）、中（10.26 g/kg）、低（5.13 g/kg）剂量组。除对照组外，其余各组均采用单次尾静脉注射链脲佐菌素（STZ，38 mg/kg）联合 28 天慢性不可预见性温和应激（CUMS）的方法建立糖尿病并发抑郁症大鼠模型。于 CUMS 造模期间灌胃给药，对照组与模型组给予等体积蒸馏水，共给药 28 天。通过检测动物空腹血糖、胰岛素、糖化血红蛋白，并结合旷场实验（OFT）、强迫游泳实验（FST）和蔗糖偏好实验（SPT）评价 ZJJF 降糖与抗抑郁的功效，同时通过苏木素-伊红（HE）染色与末端脱氧核苷酸转移酶介导的 dUTP 缺口末端标记法（TUNEL）染色评价海马组织损伤与神经元凋亡情况，免疫印迹法和实时荧光定量聚合酶链反应（RT-qPCR）法检测凋亡相关蛋白 Bax、Bcl-2、caspase-3 及 JNK/Elk-1/c-fos 信号的表达水平。（2）为进一步明确 JNK 信号在海马神经元凋亡及 ZJJF 药效中的作用，另取 50 只 SPF 级雄性 SD 大鼠，随机分为 5 组，每组 10 只：对照组、模型组、SP600125（SP6，JNK 阻断剂，10 mg/kg）组、ZJJF（20.52 g/kg）组、ZJJF（20.52 g/kg）+ Anisomycin（Aniso，JNK 激动剂，15 mg/kg）组。除对照组外，其余各组均建立糖尿病并发抑郁症大鼠模型，并在 CUMS 造模期间，灌胃给药 ZJJF，腹腔注射给药 SP6 和 Aniso，持续 28 天。通过 OFT、FST 和 SPT 评价大鼠行为学变化，并通过 HE 染色、Nissl 染色、TUNEL 染色和电镜（TEM）观察海马神经元损伤与凋亡情况，检测各组大鼠海马组织中凋亡相关蛋白及 JNK 信号通路的变化。**结果** （1）ZJJF 显著降低模型大鼠的高血糖、胰岛素及糖化血红蛋白水平（$P < 0.01$），增加自主活动水平并减少绝望样行为（$P < 0.01$），改善海马神经元病理损伤，包括增加神经元胞核数量（$P < 0.01$），减少机械细胞、空泡细胞及凋亡神经元数量（分别为 $P < 0.05$、$P < 0.01$ 和 $P < 0.01$），下调促凋亡蛋白 Bax、caspase-3 表达（$P < 0.01$），上调抗凋亡蛋白 Bcl-2 表达（$P < 0.01$），显著抑制 p-JNK、Elk-1 和 c-fos 的高表达（$P < 0.01$）。（2）SP6 能够增加模型大鼠自主活动能力（$P < 0.05$），降低绝望时间（$P < 0.05$），但对蔗糖偏好度无显著影响（$P > 0.05$），增加海马神经元尼氏小体数量（$P < 0.01$），降低 Bax、caspase-3 蛋白表达水平（分别为 $P < 0.01$ 和 $P < 0.05$），减少神经元凋亡数量（$P < 0.05$），增加 Bcl-2 表达水平（$P < 0.01$），抑制 p-JNK、Elk-1、c-fos 的高表达（分别为 $P < 0.01$、$P < 0.01$ 和 $P < 0.05$），说明糖尿病并发抑郁症大鼠海马神经元凋亡与 JNK 信号的异常激活有关；与 ZJJF 组比较，ZJJF + Aniso 组蔗糖偏好度减少（$P < 0.05$），绝望时间增加（$P < 0.01$），海马神经元损伤更加严重，Bcl-2 表达水平降低（$P < 0.01$），Bax、caspase-3、p-JNK、Elk-1 和 c-fos 蛋白表达水平升高（分别为 $P < 0.01$、$P < 0.05$、$P < 0.05$、$P < 0.01$ 和 $P < 0.05$），说明 ZJJF 抗抑郁、改善神经元凋亡及对 JNK 信号分子的调控作用均可被 JNK 激动剂所逆转。**结论** ZJJF 具有良好的降糖功效，并通过抑制 JNK 信号通路的激活减轻海马神经元的凋亡，是治疗糖尿病并发抑郁症的一种很有前景的药物。

【关键词】左归降糖解郁方；抑郁症；糖尿病；神经元凋亡；JNK 信号通路