GLP-1 plays a protective role in hippocampal neuronal cells by activating cAMP-CREB-BDNFsignaling pathway against CORT+HG-induced toxicity

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Abstract

Major depressive disorder (MDD) combined with diabetes mellitus (DM) seriously reduce the quality of life of patients, currently there is still no effective treatment. Our study explores the feasibility of GLP-1 in the treatment of major depressive disorder combined with diabetes mellitus. We aimed to assess the protective effects of GLP-1 on mouse hippocampal neuronal cell line HT22 cultured with corticosterone (CORT) and high glucose (HG). HT22 cells were cultured with CORT + HG to construct cell model of MDD combined with DM. After treatment with GLP-1, cell viability detected by CCK-8 assay, cell apoptosis/necrocytosis detected by flow cytometry/confocal laser scanning microscopy, BDNF and neurotransmitter levels in culture supernatants measured through enzyme-linked immunosorbent assay, LDH and glucose levels in culture supernatants measured via colorimetric assay, and proteins of cAMP-CREB-BDNF signal pathway measured by colorimetric assay Western blot. To construct cell model of MDD combined with DM, the ideal intervention combination are CORT 200μM and HG 50mM for 48 hours. After treatment of 50nM GLP-1 for 48 hours, the apoptosis rate and necrocytosis rate of model + 50nM GLP-1 group decreased significantly compared with the model group. The concentration of BDNF, neurotransmitter (5-HT, DA, NE), PKA, p-CREB and p-Trkb in culture supernatants of model + 50nM GLP-1 group increased significantly compared with the model group. The concentration of grouse and LDH in culture supernatants of model + 50nM GLP-1 group decreased significantly compared with the model group. GLP-1 against CORT + HG-induced toxicity by activating cAMP-CREB-BDNF signaling pathway in hippocampal neuronal cell.

Introduction

Major depressive disorder (MDD) and diabetes mellitus (DM) are both among the top 10 non-infectious diseases worldwide. According to the 2013 Global Burden of Disease Study, DM ranks seventh in disability rates among all diseases, while MDD ranks second[1]. The prevalence of MDD in DM patients is 12%-27%, which is twice that of non-diabetic people[2, 3]. Depressive symptoms have been linked to lower medication adherence of DM patients, and significantly increased the risk of DM complications[4, 5]. The medical cost of DM patients with MDD is 4.5 times that of patients with DM alone, which brings heavy medical and economic burden to individuals, families and society[6].

Currently, MDD combined with DM is treated separately for a single disease, and the interaction between drugs is unfavorable to the control of the condition. For example, tricyclic antidepressive agents (TCAs), monoamine oxidase inhibitors (MAOIs), and selective 5-HT reuptake inhibitors (SSRIs) may cause the metabolic situation to deteriorate[7]. Although blood glucose control can improve the treatment effect of MDD, antidepressant therapy alone cannot improve blood glucose continuously. Therefore, increasing the use of diabetes drug may lead to lower adherence in MDD patients[8]. For DM patients, the psychological burden caused by chronic diseases is more likely to suffer from MDD[9]. So it is important to develop a drug that simultaneously improves depressive symptoms and lowers blood glucose.

Glucagon-like peptide-1 (GLP-1) is an intestinal peptide that plays a central regulatory role through the "gut-brain" axis and is secreted by intestinal L-cells[10]. It is used in the treatment of type 2 diabetes. GLP-1 receptor is widely distributed in pancreas, stomach, duodenum, heart, kidney and central nervous system. After activation, GLP-1 enhances insulin secretion by glucose-dependent, inhibits glucagon secretion, delays gastric emptying, and reduces food intake through central appetite suppression, which reduce the blood glucose level[11]. GLP-1 can through the blood-brain barrier, bind to GLP-1 receptors distributed in the hippocampus, improve learning and memory ability, and play a neuroprotective role[12]. In this study, we explored the neuroprotective effect of GLP-1 through cAMP-CREB-BDNF signal pathway in mouse hippocampal HT22 cells, and we hope that our current results provide a reliable theoretical basis for treatment of MDD combined with DM.

Materials And Methods

Constructing cell model of MDD combined with DM

Mouse hippocampal neuronal cell line (HT22) was obtained from Yaji Biotechnology Co., Ltd (Shanghai, China). The cells were cultured in high-glucose (HG) DMEM containing 10% FBS (No. CM-M107, Procell) in 37°C cell incubator at 5% CO_2 with saturated humidity. Cell passage began when the cell fusion degree reached 90% to maintain the cells in the logarithmic growth phase for subsequent experiments. Cells were treated with various concentrations of glucose (25, 50, 75 mM) and corticosterone (CORT) (0, 100, 200μM) at different times (12, 24, 48, 72 h). The MDD combined with DM cell model was constructed by optimal compatibility group.

Cell viability detected by CCK-8 assay

Cell viability was assayed using a CCK-8 kit (Beijing TransGen Biotech Co., Ltd., Cell Counting Kit CCK, China). After trypsinization, the log phase cells were plated in a 96-well plate at 5,000 cells/well. The cells were incubated at 37°C with 5% CO_2 for 24h. The culture medium of each group was replaced with different concentrations of glucose and corticosterone, and 100μL 10% CCK-8 solution was added in medium at 12, 24, 48 and 72 h, respectively. After incubation for 1h, the OD value was determined at 450nm using a microplate meter.

Cell apoptosis was detected by flow cytometry

Cell apoptosis was assayed by the Annexin V-PE Apoptosis Detection Kit (BD Biosciences, CAT#: 559763, USA) according to the manufacturer's instructions. Cells and medium (containing apoptotic and necrotic cells generated under cultured conditions) were collected in a centrifuge tube, centrifuged at room temperature for 5 min at 1000 rpm, then discarded the supernatant. Cells were washed 2 times with prechilled PBS and the supernatant was discarded. Then added 500 μl 1×Binding Buffer and filtered with 0.22-μm filter to obtain the single cell suspension. Annexin V-PE (5μL) and 7-AAD (10μL) were added to each tube, mixed gently, and incubated at 4°C in the dark for 5 min. The apoptotic cells were detected within 30 min using a LSRFortessa flow cytometer (BD Biosciences, USA).

Necrocytosis was detected by confocal laser scanning microscopy

Necrocytosis was also detected by confocal laser scanning microscopy. The cells were seeded at a concentration of 5×10^4 cells/mL in a volume of 500μL in 24-well plates and incubated for 24 h. Cells were cultured in complete medium (control group), 50mM HG + 200μM CORT (model group) and 50mM HG + 200μM CORT + 50 nM GLP-1 (intervention group) for 48 h. Then the medium was discarded, and the cells were washed with 1 × PBS (pH = 7.4) three times. After, the cells were stained with Hoechst 33342 and Propidium Iodide (PI) for 30 min in 4°C, and rinsed twice with 1 × PBS (pH = 7.4). Cells were visualized under a confocal microscope (Leica, TCS-SP5).

Measurement of BDNF and neurotransmitter levels in culture supernatants through enzyme-linked immunosorbent assay

The BDNF, and neurotransmitter levels in cell culture supernatant were quantified by ELISA kit according to the manufacturer's protocol (BDNF: Hulti Sciences (Lianke) Biotech, Co., LTD, Hangzhou, China; 5-HT, NE, DA: Cusabio Technology Co., Ltd, Wuhan, China). The OD value was detected at 450 nm using a multi-function microplate reader (xMark™, Bio-Rad, USA).

Measurement of LDH and glucose levels in culture supernatants through colorimetric assay

The LDH was measured by Lactate dehydrogenase assay kit according to the manufacturer's protocol (Nanjing Jiancheng Corp., Nanjing, China). The OD value was detected at 450 nm using a multi-function microplate reader (xMark™, Bio-Rad, USA). The computational formula is as below:

$$\text{LDH（U/L）}=\frac{\textit{sample OD}-\textit{control OD}}{\textit{standard OD}-\textit{control OD}}\times\text{standard concentration}(0.2\mu\text{mol/L})\times1000$$

The glucose was measured by Glucose kit through glucose oxidase method (Nanjing Jiancheng Corp., Nanjing, China). The OD value was detected at 450 nm using a multi-function microplate reader (xMarkTM, Bio-Rad, USA). The computational formula is as below:

$$\text{Glucose（mmol/L）}=\frac{\textit{sample OD}-\textit{control OD}}{\textit{standard OD}-\textit{control OD}}\times\text{standard concentration}(5.5\text{mmol/L})$$

Measurement of protein of cAMP-CREB-BDNF signal pathway through colorimetric assay Western blot

Cells were washed with cold PBS and lysed with RIPA buffer, on ice and stand for 1h. Obtain the protein by centrifugation at 12,000 rpm, 4℃ for 10 min, and quantified protein concentration using a Bicinchoninic Acid assay with an Easy II Protein Quantitative kit (Beijing Transgen Biotech Co., Ltd., Beijing, China). Protein samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% nonfat milk (prepared with TBS-T) for 1 h at room temperature. Membranes were incubated overnight at 4°C primary antibody (β-actin (Sino Biological, China; 1:1000), PKA (Abcam, USA; 1: 1000), CREB (Abcam, USA; 1: 1000), p-CREB (Abcam, USA; 1:800), Trkb (Abcam, USA; 1: 1000), and p-Trkb (Abcam, USA; 1:800)). After washing, the membranes were incubated with secondary antibody at room temperature for 1h. Protein expression was visualized using chemiluminescence on the Bio-Rad system.

Statistical analysis

All quantitative data were presented as the mean ± standard deviation and performed using SPSS 25.0 software. Each experiment was repeated three times independently. One-way ANOVA was performed to analyze the differences between groups. $P < 0.05$ was considered statistically different.

Results

CORT + HG treatment construct MDD combined with DM cell model

HT22 cells were treated with 9 combinations of two compounds including 0μM, 100μM, 200μM corticosterone (CORT) and 25mM, 50mM, 75mM glucose. The neuron survival rate decreased with the increase of CORT and HG concentration. The combination of CORT 200μM and HG 50mM was an ideal intervention concentration. The reason is that cell survival in this combination was decreased to 56.92% compared with control (CORT 0μM and HG 25mM) in CCK-8 assay, and the concentration of BDNF in culture supernatants was decreased to 44.45% compared with control, and the concentration of LDH in culture supernatants was elevated by 1.61-fold versus controls (Fig. 1A-C).

CORT 200μM and HG 50mM were added into cell culture medium, cell survival and the concentration of BDNF and LDH in culture supernatants were detected at 12, 24, 48 and 72h, respectively. The results showed that the intervention effect was best at 48 h. At this time points, OD value of cell survival rate in control and model group were 0.801 ± 0.025 *v.s.* 0.441 ± 0.028, $t = 21.374$, $P < 0.001$, the concentration of BDNF in culture supernatants were (140.577 ± 13.515 *v.s.* 87.882 ± 8.463) ng/ml, $t = 5.724$, $P < 0.01$, and the concentration of LDH in culture supernatants were (130.833 ± 31.058 *v.s.* 325.00 ± 29.475) U/L, $t = -7.854$, $P < 0.01$. Therefore, HT22 cell cultured with CORT 200μM and HG 50mM for 48 hours could construct the cell model of MDD combined with DM (Fig. 1D-F).

Optimum concentration of GLP-1

To determine the optimal intervention concentration of GLP-1, we added 10nM, 20 nM, 50 nM and 100 nM GLP-1 into the cell culture medium, respectively. At 48 hours, cell viability of each group was detected by CCK8 assay, BDNF concentration in culture supernatant was detected by ELISA, and LDH concentration in culture supernatant was detected by colorimetry. Compared with the control group, the cell viability of the model group decreased to about 56.2%, model + GLP-1 10nM group decreased to 69.9% ($P < 0.0001$), model + GLP-1 20nM group decreased to 70.2% ($P < 0.0001$), model + GLP-1 50nM group decreased to 82.3% ($P < 0.0001$), model + GLP-1 100nM group decreased to 91.0% ($P < 0.001$) (Fig. 2G). The concentration of BDNF in cell culture supernatant of each group was as follows: control group 131.110 ± 14.391 ng/ml, model group 58.247 ± 4.711 ng/ml, model + GLP-1 10nM group 108.504 ± 16.555 ng/ml, model + GLP-1 20nM Group 107.538 ± 3.125 ng/ml, model + GLP-1 50nM group 135.269 ± 9.264 ng/ml, model + GLP-1 100nM group 152.634 ± 5.758 ng/ml (Fig. 2H). The concentration of LDH in cell culture supernatant of each group was 133.333 ± 15.927 U/L in control group, 302.682 ± 28.743 U/L in model group, 282.758 ± 33.155 U/L in model + GLP-1 10nM group and 20nM in model + GLP-1 group 239.080 ± 30.149 U/L, model + GLP-1 50nM group 177.887 ± 12.661 U/L, model + GLP-1 100nM group 200.766 ± 36.661 U/L(Fig. 2I). Combined with cell survival rate, BDNF and LDH, we confirmed that 50 nM was the optimal intervention concentration of GLP-1.

Protective effect of GLP-1 on CORT + HG-induced apoptosis and necrosis of HT22 cells

We performed flow cytometry and confocal laser scanning microscopy to assess the effects of GLP-1 on the apoptosis and necrosis of HT22 cells cultured under the CORT + HG conditions. Flow cytometry results showed that compared with the control group, the apoptosis rate of model group increased significantly (2.43% *v.s.* 11.0%). After GLP-1 treatment, apoptosis rate decreased to 5.76% (Fig. 3A). Cell necrosis was observed using confocal laser scanning microscopy after Hoechst 33342 and PI staining. Since Hoechst 33342 can penetrate through cell membranes, the fluorescence intensity of apoptotic cells was obviously enhanced compared with normal cells. In contrast, PI cannot penetrate the cell membrane and cannot stain normal cells or apoptotic cells with intact cell membranes. Therefore, the differential staining effect of Hoechst 33342 and PI can detect normal cells (weak red and weak blue fluorescence) and necrotic cells (strong red and strong blue fluorescence). Our results showed that compared with the control group, Hoechst 33342 and PI fluorescence intensity were both significantly increased in model group. After GLP-1 (50 nM) treatment, the two-fluorescence intensity was decreased (Fig. 3B). Thus, GLP-1 treatment has a protective effect against apoptosis and necrotic of HT22 cells under the CORT + HG conditions.

Effects of GLP-1 on the neurotransmitter and glucose in the culture supernatant of HT22 cells cultured with CORT + HG

We used the ELISA method to detect the effects of GLP-1 on the neurotransmitter of CORT + HG cultured HT22 cells. The results revealed that the concentration of 5-HT, NE and DA in culture supernatant of

model group was lower than that of control group (5-HT: 2270.95 ± 83.02 *v.s.* 2629.59 ± 120.33 pg/ml, *n* = 3, *P* 0.01; NE: 41.10 ± 2.54 *v.s.* 63.03 ± 9.98 pg/ml, *n* = 3, *P* 0.05; DA: 17.80 ± 1.76 *v.s.* 32.26 ± 9.07 pg/ml, *n* = 3, *P* 0.05). After 50nM GLP-1 treatment, the concentration of neurotransmitter increased significantly, 5-HT increased to 2738.84 ± 107.09 pg/ ml, NE increased to 81.64 ± 7.33 pg/ml, DA increased to 51.72 ± 2.58 pg/ml. Compared with the model group, statistically significant differences (*P* 0.01; *P* 0.001; *P* 0.001). (Fig. 4A-C)

The glucose oxidase method was used to detect the glucose content in the supernatant. The concentration of glucose in supernatant of model group was significantly higher than that of control group (8.28 ± 0.53 *v.s.* 4.33 ± 0.55 mmol/L, n = 3, *P* 0.0001). After 50nM GLP-1 treatment, glucose concentration decreased to 6.347 ± 0.73 mmol/L, which was statistically significant compared with the model group (*P* 0.01) (Fig. 4D). Since the model group cells were cultured in high glucose medium, it was reasonable that the glucose content in the supernatant was significantly increased. However, glucose concentration decreased significantly after GLP-1 intervention, suggesting that GLP-1 can effectively improve glucose metabolism of HT22 cells.

GLP-1 reversed the cAMP-CREB-BDNF signaling pathway inhibition in HT22 cells caused by CORT + HG

We used the Western blot assay to detect key members of the cAMP-CREB-BDNF signaling pathway in the HT22 cells. As shown in Fig. 5B, β-actin expression was used as internal standard, the relative expression of PKA protein in the control group, model group and GLP-1 group was 0.50 ± 0.01, 0.27 ± 0.04 and 0.473 ± 0.06 respectively. Compared with model group, the ratio of PKA protein to β-actin is the difference between the control group and GLP-1 intervention group (*P* 0.001). The ratio of p-CREB/CREB in the control group, model group and GLP-1 group was 0.444 ± 0.014, 0.306 ± 0.017 and 0.431 ± 0.015 respectively. Because of CORT + HG culture, the ratio of p-CREB /CREB was significantly lower than that in control group(*P* 0.0001), and after treatment with 50 nM GLP-1 the ratio was comparable with the control group (Fig. 5C). Similarly, HG + CORT culture cause the p-TrkB/Trkb ratio in the model group was significantly lower than that in the control group (0.35 ± 0.03 *v.s.* 0.51 ± 0.09, *P* 0.05). After treatment with 50 nM GLP-1, the p-TrkB/Trkb ratio increased significantly, and the differences were statistically significant compared with the model group (0.46 ± 0.04 *v.s.* 0.35 ± 0.03, *P* 0.05) (Fig. 5D). These data indicate that GLP-1 can reversed the cAMP-CREB-BDNF signaling pathway inhibition in HT22 cells caused by CORT + HG.

Discussion

The complex array of multidirectional linkages between MDD and DM raises the possibility of a shared etiology[13]. Hippocampus is a part of the limbic system responsible for memory and emotion processing, which is both associated with MDD and DM. Neurogenesis is the proliferation of new neurons in the brain. Studies have shown that hippocampal neurogenesis-deficient mice showed symptoms of

anhedonia, suggesting a role for the hippocampus in mediating depressive symptoms[14]. Both MDD and DM are associated with hippocampal volume reduction[15, 16]. The volume of gray matter in the hippocampus begins to decrease in early-life of MDD patients, and changes in other brain regions begin to occur as the disease progresses[17]. Likewise, neuroimaging studies have found that impaired functional connectivity between the left hippocampus and multiple core brain regions of the default network in DM patients[18]. Therefore, neuroplastic changes in the hippocampus are important to understanding the link between MDD and DM. We stimulated mouse hippocampal neuronal cell line (HT22 cell) with CORT and HG to simulate hippocampal neuron damage in the comorbidity of MDD and DM. To be close to the real clinical situation, this study used BDNF as the evaluation index of MDD and glucose as the evaluation index of DM to verify that the model was successfully established. In addition, LDH release is related to cell membrane integrity, and can leak into cell culture supernatant when cell membrane is damaged or the permeability of cell membrane is increased, and the release amount is positively correlated with the degree of cell damage. Therefore, detection of LDH concentration in culture supernatant is one of the evidence to judge cytotoxicity[19].

GLP-1 is an intestinal peptide with extensive pharmacological effects. In addition to treating type 2 diabetes and obesity, it also has cardio- and neuroprotective effects. It affects learning, memory, and reward behavior by reducing inflammation and apoptosis[20]. Clinical studies have found that in addition to treatment of diabetes and obesity, GLP-1 receptor agonists has also been shown to alleviate depressive symptoms[21]. Our previous study found that the plasma and fecal GLP-1 levels of the MDD group were lower than those of the control group[22]. In animal experiments, GLP-1 receptor agonist liraglutide reverses the metabolic abnormalities and behavioral depression caused by long-term administration of atypical antipsychotics[23]. These previous studies all indicate that GLP-1 could be used as a potential therapy for depression in the context of diabetes. In our study, CORT + HG successfully induced the cell model of MDD combined with DM, resulting in decreased survival rate, increased apoptosis and necrosis, decreased glucose utilization, and decreased BDNF, 5-HT, DA and NE production ability of HT22 cells. GLP-1 treatment effectively reversed the activity of damaged HT22 cells, reduced apoptosis and necrosis, effectively increased the level of BDNF, 5-HT, DA and NE in the culture medium and decreased the concentration of glucose in the culture medium, providing more evidence for GLP-1 treatment of MDD combined with DM.

cAMP-CREB-BDNF signaling pathway is one of the important factors in neurogenesis and neuronal cell repair, which is related to learning, memory and cognitive function[24, 25]. cAMP exerts its effects mainly through the stimulation of PKA, which phosphorylates CREB. When CREB is activated, BDNF, a powerful trophic factor that affects synaptic plasticity and neuronal shape, is produced[26, 27]. MDD patients who died by suicide had reduced cAMP levels in the prefrontal cortex[28]. Postmortem studies showed that patients with significant depression had low levels of phosphorylation level of CREB in the hippocampus[29]. In chronic unpredictable stress animal model, CREB expression was inhibited in hippocampal CA1 region, but CREB levels recovered after antidepressant treatment[30].Through

modulation of cAMP-CREB-BDNF and other similar signaling pathways, numerous neuroprotective agents have been developed against neurodegenerative and neurobehavioral disorders[31]. In our study, the expression of PKA, p-CREB and p-Trkb (BDNF-specific receptor) were significantly increased after the treatment of GLP-1 to the culture medium of HT22 cells which damaged by CORT + HG, suggesting that GLP-1 could up-regulate the cAMP-CREB-BDNF signaling pathway. This may be the underlying mechanism by which GLP-1 exerts protective effects on neuronal cell. These novel results provide us with new insights into the molecular role of GLP-1 in hippocampal cells. Further studies are warranted to confirm these findings and explore the feasibility of GLP-1 as a treatment for MDD combined with DM.

Declarations

Author contribution QM, LW and XL contributed to study design, experimental operation, data analysis, and draft manuscript. ZA, XL, LZ, PY and LJ contributed to data analysis. RC contributed to cell culture. BX and QY contributed to study design, study supervision, and manuscript preparation.

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Availability of Data and Materials The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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Figures

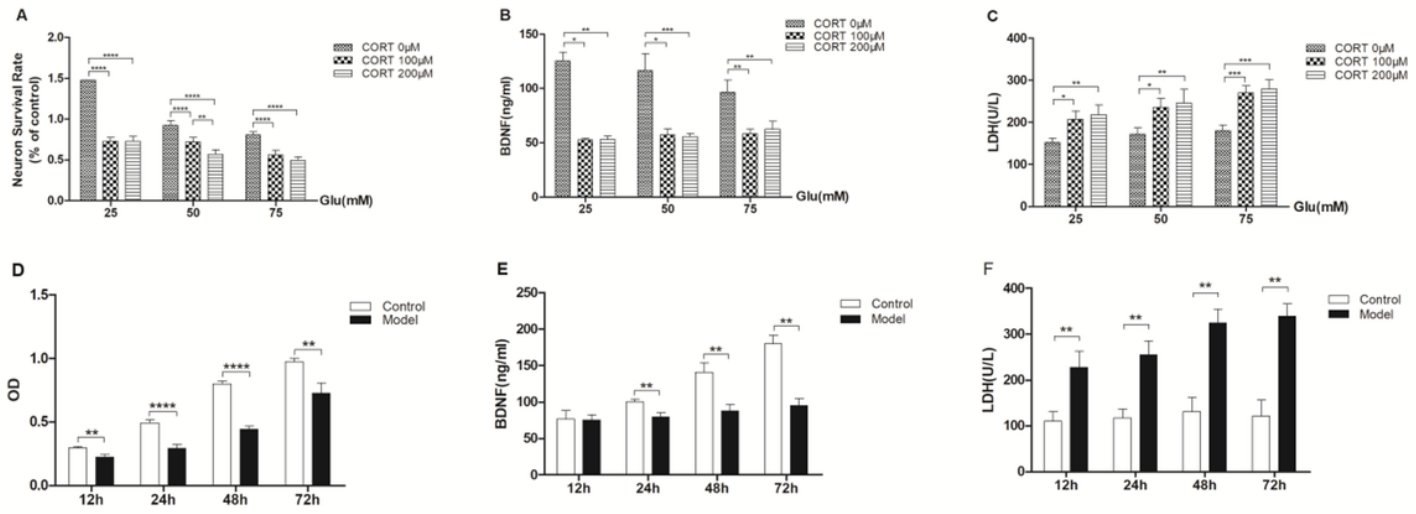

Figure 1

The cell survival rate, concentration of BDNF and LDH in culture supernatants of HT22 cells with different CORT and HG concentrations and time. (n=3 per group). Control: HG 25 mM + CORT 0μM; Model: HG 50 mM + CORT 200μM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

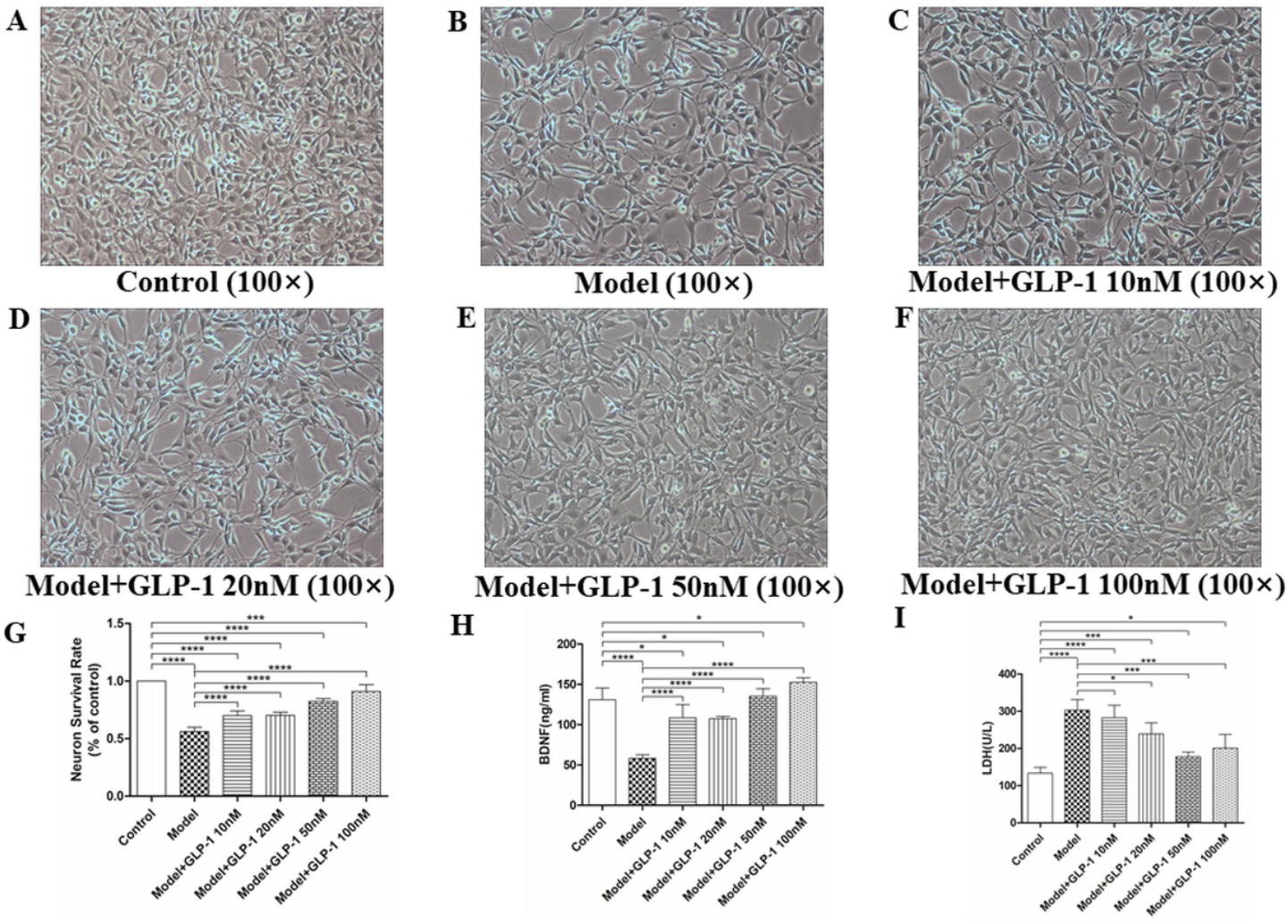

Figure 2

Effects of different GLP-1 concentration on HT22 cell survival rate, and BDNF and LDH concentration in culture supernatants. (n=3 per group). Control: HG 25 mM + CORT 0μM; Model: HG 50 mM + CORT 200μM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

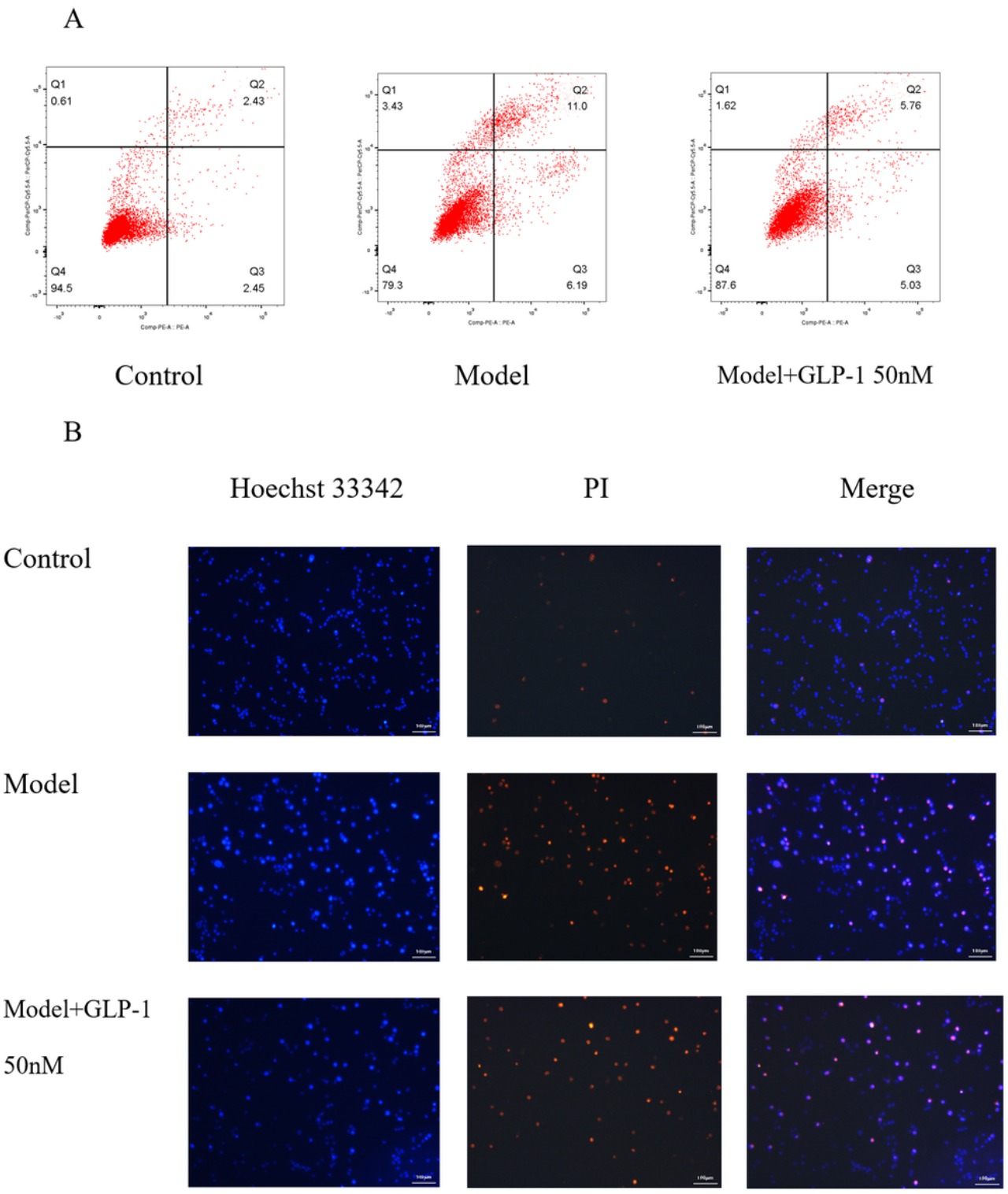

Figure 3

Effects of GLP-1 on the HT22 cells cultured under the CORT+HG condition. (n=3 per group).

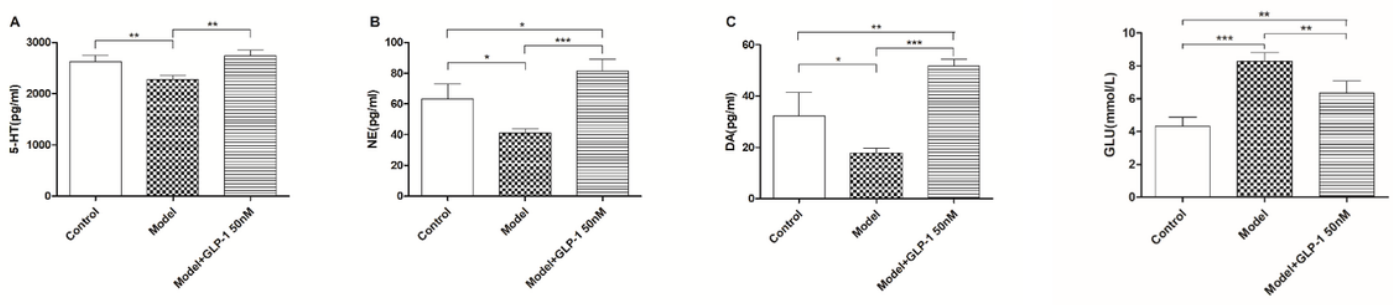

Figure 4

Effects of GLP-1 on the neurotransmitter and glucose in the cultre supernatant of HT22 cells cultured with CORT + HG. A-C The ELISA method was used to detect the effects of GLP-1 on 5-HT(A), NE(B) and DA(C) of HT22 cells cultured with CORT + HG. **D** Using glucose oxidase method to detect the glucose content of HT22 cells in the supernatant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

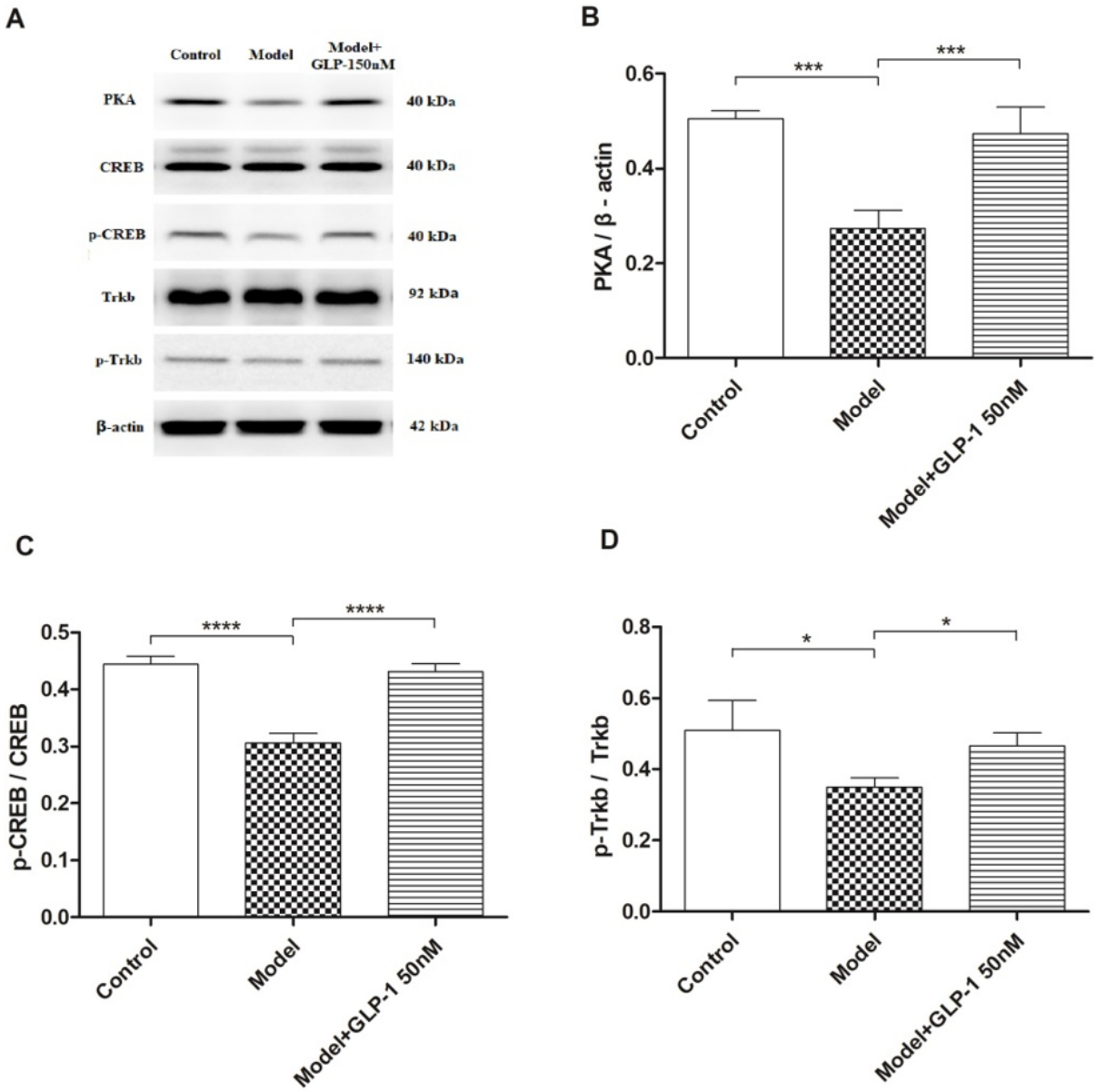

Figure 5

Expression of key members of the cAMP-CREB-BDNF signaling pathway in the cell model of depression with combined diabetes mellitus. A PKA, CREB, p-CREB, Trkb, p-Trkb and β-actin expression levels in HT22 cells were detected using western blotting. **B-D** Bar chart showing the ratio of PKA/β-actin (B), p-CREB/CREB (C) and p-Trkb/ Trkb (D) in HT22 cells. **P* < 0.05, ****P* < 0.001, *****P* < 0.0001.