

TGF-beta/TGF-beta RII/CLC-3 axis promotes cognitive disorders in diabetes

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1. ABSTRACT

Transforming growth factor beta (TGF-beta) and Chloride channel-3 (CLC-3) are critical for inflammatory response, cellular proliferation and apoptosis in hippocampus neurons. However, the relationship between CLC-3 and TGF-beta/TGF-beta Receptor II (RII) pathway in diabetic encephalopathy (DE) is unknown. In this study, both diabetes rat model and diabetes cell model were employed to elucidate the mechanisms involved. The increased expressions of CLC-3 and TGF- beta RII with cognitive impairment were observed in diabetic rats. The most obvious reduction on the survival of HT22 cells was at 10 ng/ml or 15 ng/ml TGF- beta stimulation, while the expressions of CLC-3 and TGF-beta RII were

significantly increased under high glucose condition. Moreover, the study showed that CLC-3 antagonists had no apparent effect on up-regulated TGF- beta RII, but TGF- beta 1 inhibitors could reduce the up-regulated CLC-3 under high glucose. Results from the present study indicated that CLC-3 and TGF- beta signals might be related to cognitive disorders. The CLC-3 might be modulated by TGF- beta /TGF- beta RII signaling pathway during the development of DE.

2. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder with the characteristic of increased blood

glucose level, which is caused by the impaired insulin secretion, insulin resistance, or both. DM can cause long-term damage of eye, liver, kidneys, nerves and blood vessels, which may lead to the neurodegenerative disease (1-3). The development of DM is correlated with the increased risk of brain diseases, especially a growing body of evidence suggests that DM could increase the risk of cognitive decline and Alzheimer's disease (AD) (4). DM can lead to cognitive impairment disease including diabetic encephalopathy (DE), i.e. DM caused degeneration of nervous system which eventually leads to neurological disorders (5-8).

TGF- β , a cytokine, is named according to the function of transforming the phenotype of fibroblasts in 1981 (9). TGF- β participates in basic biological function of cells, such as cellular proliferation, migration, differentiation, survival, apoptosis, the formation of extracellular matrix and angiogenesis (10,11). In recent years, the study confirmed that TGF- β could inhibit the proliferation of neural stem cells in the adult brain and neurogenesis (12). Research has shown that high glucose can increase the expressions of TGF- β RI and TGF- β RII, which could rapidly activate the TGF- β signaling pathway (13,14).

CLC is a pivotal biology anion channel which participate in the regulation of cellular excitability, transporting across epithelial cells, cell volume regulation and organelles acidification, cellular immune response, cell proliferation, differentiation, apoptosis and other physiological processes (15,16). CLC-3 is an important chloride ion channel proteins included in the regeneration process of a variety of tissues and cells. Studies have shown that CLC-3 was highly expressed (17.1.8) in the neurons of central nervous system, such as hippocampus and olfactory bulb. Our previous study has revealed that the high glucose condition could induce apoptosis of neuronal HT22 cells with the highly expression of CLC-3 (19).

Both TGF- β 1 and CLC-3 that are highly expressed under high glucose can actively participate in cell proliferation and play an important role in regulating the survival of hippocampus neurons. Whether CLC-3 was a downstream signaling molecule of the TGF- β 1 signaling pathway in adult neurogenesis and the DE development was still unclear. Hence, the relationship between CLC-3 and TGF- β in diabetic cognitive disorders need to be elucidated. In this study, we detected the expressions of CLC-3 and TGF- β RII in diabetic rat model and treated HT22 cells with high concentration glucose to analyze their relations with neurogenesis, which could provide clues to explore the possible mechanisms of CLC-3 and TGF- β involved in the nervous system damage under high glucose condition.

3. MATERIALS AND METHODS

3.1. Animals and cells

Sprague-Dawley rats (Male, 200-250 g) were obtained from Animal Center of Air Force Medical University, China. The animals were kept and maintained under standard environmental conditions (12 h day: 12 h night cycle), temperature ($22 \pm 2^\circ\text{C}$) and relative humidity of 50%. Water and industrialized dry food were available with adding libitum. The neuronal HT22 cells were generous gift from Department of Anesthesia, Tangdu Hospital, Air Force Medical University, China. The study was approved by the Medical Ethics Committee of Tangdu Hospital, Air Force Medical University, China.

3.2. Diabetic animal model

Animals were acclimatized to standard husbandry conditions for 1 week to eliminate the effect of stress prior to initiation of the experiments. Fifteen rats were selected randomly as control group (C), and nineteen rats were applied to induce diabetes. Rats were fasted overnight more than 12 h and injected with a single intraperitoneal injection of streptozotocin (STZ, Sigma-Aldrich Chemical Company, Missouri, St Louis, USA) at a dose of 60 mg/kg-body weight in freshly prepared 0.1. M cold citrate buffer (pH 4.5). On the third day, DM development in the experimental groups was confirmed by measuring fasting blood glucose (FPG) levels of blood samples from the tail vein of overnight fasted rats using glucose meter (maximum blood sugar value was 33.3 mmol/L, more than 33.3 mmol/L showed High which was recorded as 33.3 mmol/L). Rats exhibiting FPG of 16mmol/L or higher were considered to be diabetic and chosen for the experiments. This day was defined as the first day of the experiment. Seven days later, the STZ-induced diabetic rats whose random blood glucose levels were greater than 16 mmol/L were considered as stable diseases.

3.3. Monitoring the body weights and blood glucose levels

The mental state, coat color, eating, urine output, etc of the rats were observed and recorded. The body weights and blood glucose levels of all the rats were monitored weekly during the experimental period.

3.4. Morris water maze tasks

Morris water maze tasks were performed in a circular swimming pool made of blackpainted fiberglass, with 1.2 m internal diameter and 0.8 m height, and filled with water at 25°C to a depth of 0.42 m at 5 weeks after diabetes induction. The target platform (10×10 cm) was made of transparent plexiglas

and was submerged 1-1.5 cm beneath the surface of the water. The morris water maze was divided into four quadrants by four points equally distributed on the edge of the pool. The directional navigation experiments and space exploration experiments were done accordingly: (1) directional navigation experiment: the rats were left in the pool facing the wall randomly selected the entry point. If the rat did not find the platform during a period of 60 s, it was gently guided to the platform. The rat was allowed to remember the platform for 10 s after escaping to it and was then removed from the pool. The protocol consisted of four training days, four consecutive trials per day. (2) space exploration experiment: the test consisted of a single probe trial where the platform was removed from the pool and each rat was allowed to swim for 60 s on the fifth day. The whole experiment process is monitored by camera system, and the experimental data such as latency period, swimming distance, swimming speed, number of crossing the platform, initial crossing platform time and platform quadrant swim distance were recorded.

3.5. The levels of serum TGF- β 1

The rats were subjected to anesthesia with intraperitoneal (i.p.) injection of chloral hydrate (3.5 mL/kg of 10%) the next day followed the morris water maze tasks and 5 ml blood was collected from abdominal aorta of each rat. Then the blood samples were centrifuged at a rate of 3000 G/min at 4°C to collect the plasma. The plasma was used to detect the level of TGF- β 1 using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacture's protocol and the automatic microplate reader.

3.6. Osmotic Control

HT22 cells were cultured in high glucose dulbecco's modified eagle medium (DMEM, HyClone, Utah, USA), which contained 25 mM glucose, 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, California, USA) and maintained under 5% CO₂ at 37°C and differentiated in NeuroBasal medium (Invitrogen, Carlsbad, CA) containing 2 mmol/L glutamine and 1× N2 supplement for 24 h before use. Optimal survival rate and neurite growth of hippocampal neurons require 25 mM basal glucose (20), reflecting the fact that neurons have high metabolic rates. Neurobasal medium containing 25 mM glucose (control condition) meets these metabolic requirements.

HT22 cells were cultured in a petri dish *in vitro* and medium were replaced with a fresh culture medium containing additional 25 mM glucose (Sigma, Missouri, USA) (total osmolarity from glucose + glucose = 25 + 25 = 50 mM; glucose high osmolarity group), 5 mM glucose (total osmolarity from glucose + glucose = 25 + 5 = 30 mM; glucose low osmolarity group), 25 Mm mannitol (total osmolarity from glucose + mannitol

= 25 + 25 = 50 mM; mannitol high osmolarity group) or 5 mM mannitol (total osmolarity from glucose + mannitol = 25 + 5 = 30 mM; mannitol low osmolarity group). No mannitol or additional glucose was added to control group (total osmolarity from glucose = 25 mM; control). The cells were cultured for 24, 48, and 72 h before being subjected to cell counting kit-8 (CCK-8, Dojindo Laboratories) assay to assess the change in cell viability.

3.7. CCK-8 proliferation assay

Cells were plated onto 96-well plates (4×10³ cells/well) in a quadruplicate pattern and cultured with different concentrations of glucose and TGF- β . At 0, 24, 48 and 72 h, 100μL fresh medium containing 10 μL of CCK-8 solution was added to culture medium for another 3 h at 37°C. The absorbance at 490nm was measured using a microplate reader (Molecular Devices). In each group, six samples were measured to get the mean value and standard deviation. The cell viability was calculated by dividing the optical density (OD) value of experimental group with mean OD of normal control group and multiplying by 100.

3.8. Western blot analysis

After morris water maze tasks, the rats were subjected to anesthesia with intraperitoneal (i.p.) injection of pentobarbital (50 mg/kg). The separated hippocampus tissues and cell samples at 24 h from different groups were homogenized in radio immunoprecipitation assay (RIPA) buffer, supplemented with a cocktail of protease inhibitors (Sigma Chemical Co. St. Louis, MO, USA). After 15 min at 4°C, homogenates were gently vortexed for 15 seconds and centrifuged at 4°C at 12,000 G for 10 min; the supernatant was harvested and stored at -80°C for further use. The total protein levels were determined using bicinchoninic acid (BCA) assay. Proteins samples were separated in SDS, polyacrylamide gel (SDS-PAGE). After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Merck, Darmstadt, Germany) for 60 min at 15 V in a Bio-Rad Trans-Blot SD system (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% non-fat dry milk in tris-buffered saline with tween-20 (TBST) (pH 7.4) for 60 min at room temperature and then incubated overnight at 4°C with a 1:1000 dilution of CLC-3 primary antibody (Abcam, Cambridge, UK) and 1:1000 dilution of TGF- β primary antibody (Abcam, Cambridge, UK). Membranes were washed and then incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (1:2000). Immunoblots were visualized using electrochemiluminescence (ECL) by enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences, Buenos Aires, Argentina). Autoradiographical signals were quantified by densitometry using image quant software and adjusted by the density of β -actin.

3.9. Immunohistochemistry

Rats were anesthetized and intracardially perfused with saline solution, followed by a fixative solution containing 4% formaldehyde in 0.1 M PBS, pH 7.4. Then, the brain tissues were carefully removed and immersed for 24 hours in the same fixative. After dehydration, brain tissues were embedded in paraffin wax and sectioned into 5 μ m. Slices were deparaffinized using xylene and rehydrated through a graded series of decreasing concentrations of ethanol.

Antigen retrieval was performed by heating (90°C) slices for 20 minutes in citrate buffer (pH 6.3.) and then endogenous peroxidase was quenched by treating slices with 3% H₂O₂ for 10 min. Slices were blocked with 10% normal goat serum in 0.01 M PBS for 1 h and then treated with 1:500 dilution of CLC-3 and TGF- β RII primary antibody (Abcam, Cambridge, UK) at 4°C overnight. Slices were washed in PBS and then probed with biotin-labeled secondary antibody (anti-rabbit IgG, dilution 1: 100) for 30 min at room temperature. Then after thorough rinse in PBS, slices were treated with the avidin-peroxidase complex (Vectastain ABC Elitekit) and visualized with 3-3'-diaminobenzidine-4-HCL (DAB)/H₂O₂ (Imm-PACT-DAB) (Dako, Carpinteria, CA, DAB chromogen system, Code K3468). The sections were then lightly counter stained with hematoxylin, dehydrated through increasing concentrations of ethanol, cleared in xylene, and sealed with neutral gum. Specimens were examined and photographed with a Nikon FXA microscope, and digital images were recorded using a Nikon DXM1200F digital camera.

3.10. Statistical Analysis

All the experiments were performed with a sample size of six ($n = 6$) and data are presented as the means \pm standard deviation, unless specified. Statistical analysis was performed using the SPSS 11.0 software package. The comparisons of the weight and blood sugar levels, serum TGF- β 1 levels and data of the water maze were performed using unpaired Student's *t*-test. Comparison between groups was performed using variance (ANOVA), followed, when necessary, by Newman-Keuls test. The qualitative data were compared using the χ^2 test. $P < 0.05$ was considered to be significant.

4. RESULTS

4.1. Modeling results and cognitive impairment of DM rats

The sixteen of the remaining nineteen rats were successful modeling by injecting STZ for diabetic model with only one DM rat died during the experimental process and the success rate of DM model was about 80%. The rats of DM group were apathetic, slowed in action, dull and dirty, also they ate less, but drank more

water and had increased urine volume. The rats were weighed and the blood glucose levels were measured weekly. The weights of the control group had a rising trend, while that of the DM group had a declining trend (Figure 1A). Besides, diabetic rats maintained higher glucose levels than that of the control group (Figure 1B).

Morris water maze tasks revealed that the DM rats took a longer time to the platform and the number of DM rats crossing the platform was less at the fifth day (Figure 1C, D and E). Moreover, there was no apparent difference between the average swimming speed of the two groups.

4.2. The expressions of CLC-3 and TGF- β RII in DM Rats

To investigate the expressions of CLC-3 and TGF- β RII in DM rats, the total proteins of hippocampus tissues were extracted and subjected to western blot analysis. Our data showed that DM rats had a significantly increased expressions in both CLC-3 and TGF- β RII (Figure 2A and 2B).

In addition, immunohistochemical analysis was used to detect the expressions of CLC-3 and TGF- β RII in hippocampus CA1 area of the brain tissues. The hippocampus CA1 area of the DM group showed the marked disorganization with the loss of small pyramidal cells and the elevated expression of both CLC-3 and TGF- β RII in pyramidal cells (Figure 2C). Besides, the data revealed that the expressions of both CLC-3 and TGF- β RII in glial cells and neurons of molecular layer (ML) were also upregulated in DM group, suggesting a possible coordinated expression between CLC-3 and TGF- β RII.

Additionally, as a ligand to TGF- β RII, the serum levels of TGF- β 1 in DM rats were also determined. Our data demonstrated that the serum TGF- β 1 levels in DM rats significantly increased as compared with the control group, which is parallel with the expression levels of TGF- β RII. Therefore, it might be meaningful to use serum TGF- β 1 as a biomarker for DE diagnosis and treatment.

4.3. Glucose upregulated CLC-3 and TGF- β RII expression levels in HT22 cells

To verify the expressions of CLC-3 and TGF- β RII *in vitro*, we next employed the HT22 cells for the establishment of diabetes cell model. As the osmolarity would affect the survival of cells, we first assessed the cell viability under diverse conditions. The results demonstrated that cell survival in glucose low osmolarity group (GL), mannitol low osmolarity group (ML), mannitol high osmolarity group (MH) and the control group had no significant difference while the cell survival rate in glucose high osmolarity group (GH) was obviously lower than that of other groups, especially at

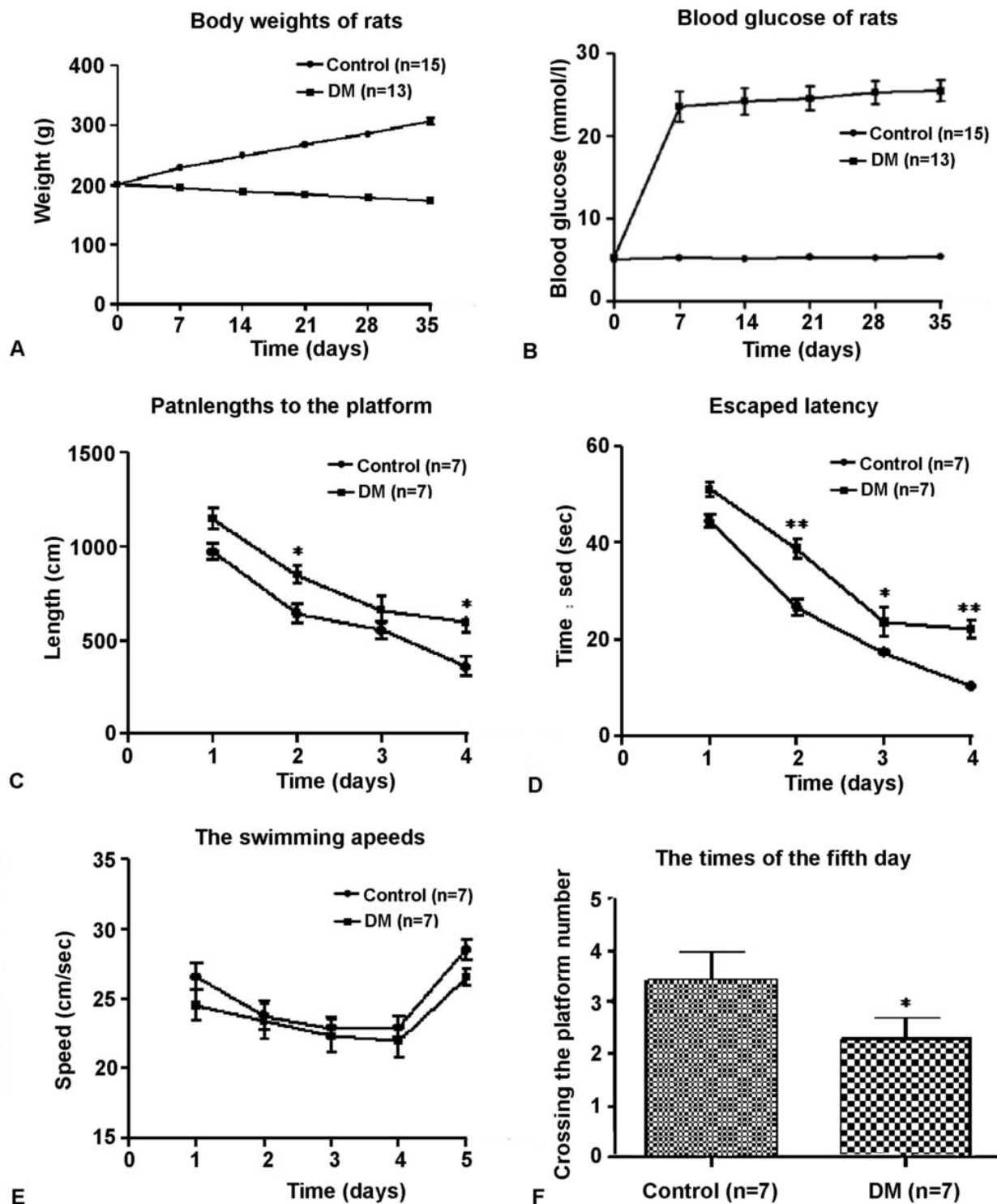


Figure 1. The weight and blood sugar levels between the induced diabetes mellitus (DM) rat models and the control, as well as the behavior analyses. (A) Comparison of the weight between diabetes mellitus (DM) group and control group of rats. (B) The blood sugar levels. (C) The pathlength to the platform of the morris water maze training period. (D) The mean escape latency during the visible platform (days 1–4) and hidden platform (days 5). (E) The number of platform area crossing. (F) Swimming speed. The data are represented as mean \pm standard error and subjected to Student's t-test analysis. * $p < 0.0.5$; ** $p < 0.0.1$.

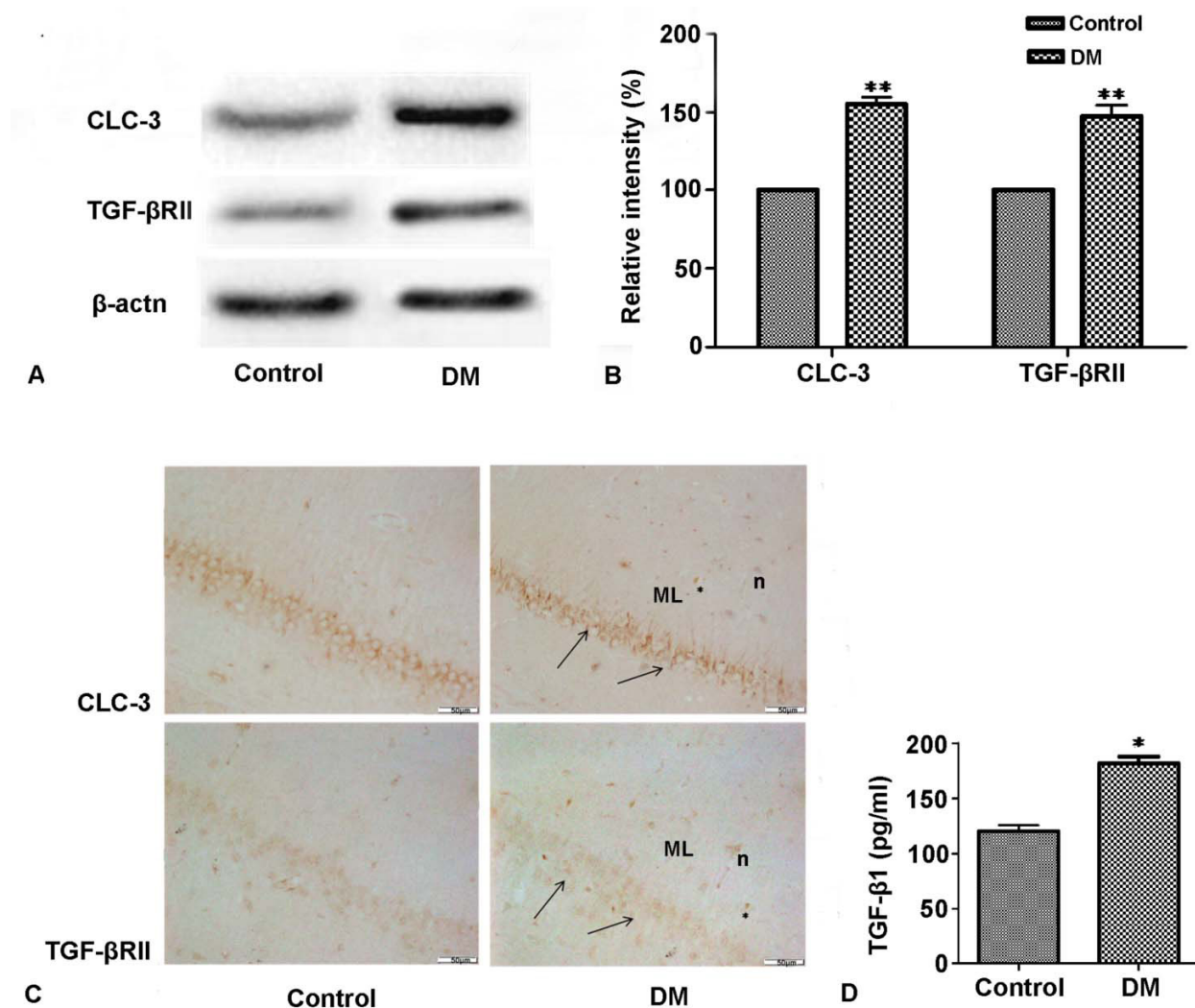


Figure 2. The expressions of chloride channel-3 (CLC-3) and transforming growth factor beta receptor II (TGF-βRII) in hippocampus of DM rats and the levels of serum transforming growth factor beta 1 (TGF-β1) in DM rats. (A) Representative bands of CLC-3 and TGF-βRII expression in western blot and (B) densitometry analysis of CLC-3 and TGF-βRII levels in the hippocampus. (C) The expressions of CLC-3 and TGF-βRII in hippocampal CA1 area of diabetic rats in immunohistochemistry. Original magnification x 100. (D) Comparison of serum TGF-β1 levels in rats. The statistical analysis was performed by ANOVA with post hoc Student-Newman-Keuls test. Data in D were analyzed by Student's t-test. * $p < 0.05$; ** $p < 0.01$.

24 h (Figure 3A). CCK-8 assay revealed no noticeable changes in HT22 cell survival in response to change in the osmolarity while reproducing a significant decrease under high glucose group (Figure 3B). These data ruled out the possibility that the osmolarity would affect the survival of cells. Also, the increase in osmolarity failed to elicit changes in the CLC-3 and TGF-βRII expressions in HT22 cells, while the same was found significantly increased in glucose high osmolarity group by western blot assay (Figure 3C and 3D). These results indicate that the level of glucose but not the level of osmolarity is the main cause of the observed phenomenon.

4.4. The effects of TGF-β on the expressions of CLC-3 and TGF-βRII in diabetes cell models

To determine the effects of TGF-β on the expressions of CLC-3 and TGF-βRII in diabetes

cell models, we first evaluated the appropriate concentrations of TGF-β. The cells were cultured with 25 mM glucose and different concentrations of TGF-β (0, 5, 10, 15 and 30 ng/ml). CCK-8 assay revealed that TGF-β had no significant effect on the proliferation of cells in the absence of glucose, however, the results differed under the high glucose condition (Figure 4A). Further study illustrated that the proliferation of cells was reduced under the condition of 25 mM glucose and TGF-β (10 and 15 ng/ml) for 24 h (Figure 4B). Moreover, the western blot demonstrated an increase in the protein expressions of both CLC-3 and TGF-βRII in 25 mM glucose and TGF-β (10 and 15 ng/ml) stimulation groups at 24 h (Figure 4C and 4D). Herein, our data indicated that TGF-β regulated the expressions of both CLC-3 and TGF-βRII in the diabetes cell models, which was consistent with the results of the animal experiments aforementioned.

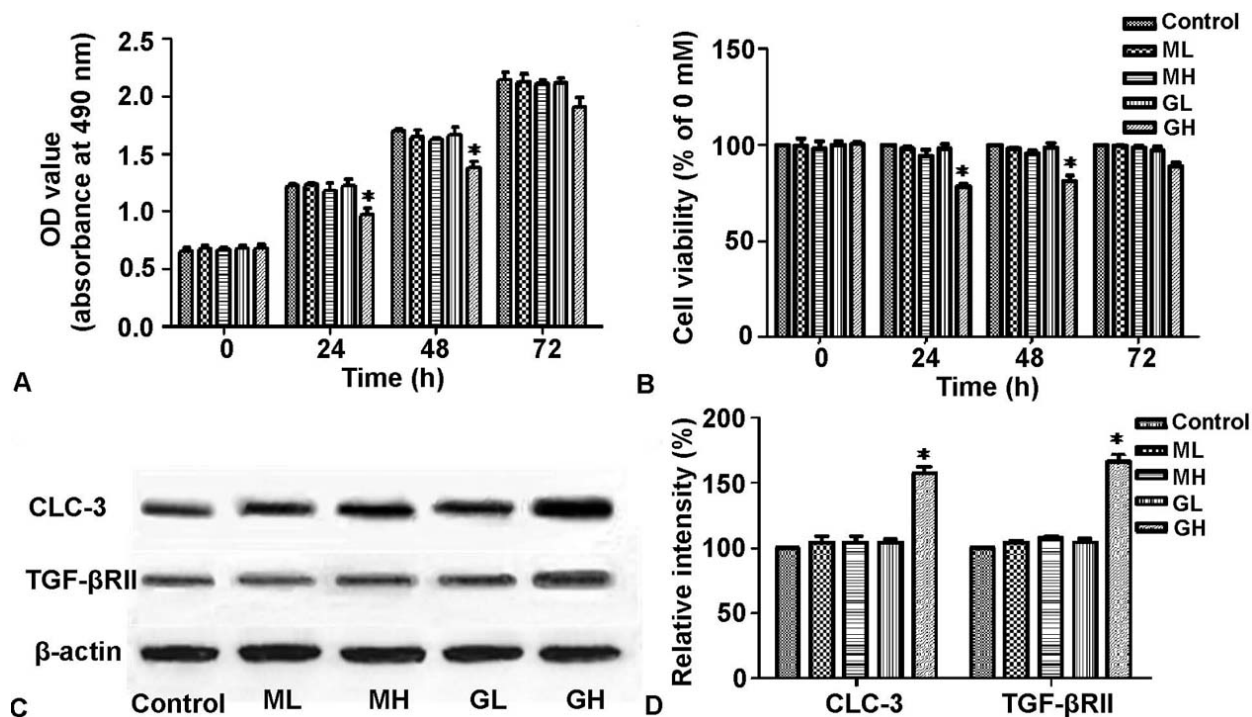


Figure 3. The optical density (OD) value, the relative proliferation rate and the expressions of CLC-3 and TGF- β RII in the cell modeling including osmotic control. (A) OD value and (B) relative proliferation rate of CCK-8 assay. (C) Representative bands of western blot and (D) densitometry analysis of CLC-3 and TGF- β RII levels in the cells of control, mannitol low osmolarity (ML) group, mannitol high osmolarity (MH) group, glucose low osmolarity (GL) group and glucose high osmolarity (GH) group. The statistical analysis was performed by ANOVA with post hoc Student-Newman-Keuls test. * $p < 0.05$.

4.5. TGF- β /TGF β RII signaling pathway modulated the expression of CLC-3

To investigate whether TGF- β /TGF β RII signaling pathway regulated the expression of CLC-3. The cells were treated with 5 μ M TGF- β inhibitor ITD-1 for 1 h, and then 25 mM glucose was added to culture medium for 24 h. The western blot analysis demonstrated that TGF- β inhibitor ITD-1 could reduce the increased expressions of both CLC-3 and TGF- β RII when treated with TGF- β under high glucose condition (Figure 5A and 5B). Thus, the results suggested that TGF- β was a true regulator to modulate both the expressions of CLC-3 and TGF- β RII.

On the other hand, to further study whether CLC-3 expression could also affect the TGF- β /TGF- β RII signaling pathway, we used the a chloride channel blocker 5-nitro-2,3-(phenylpropylamino)-benzoic acid (NPPB) to block the endogenous anion channels which can be upregulated by CLC-3. NPPB is a CLC-3 related and nonspecific chloride channel blockers (21). NPPB and CLC-3 siRNA may have similar blocking effects on CLC-3 (22,23,19). The cells were culture with added 25 mM glucose and then treated with 0.1. M NPPB for 24 h. The western blot analysis revealed that the CLC-3 inhibitor NPPB could not significantly alter the increased expression of TGF- β RII by TGF- β under high glucose condition. Taken together, our data indicated that TGF- β /TGF- β RII signaling pathway

could efficiently regulate the expression of CLC-3 in diabetes cell models, but CLC-3 antagonists had no apparent effect on up-regulated TGF- β /TGF- β RII signaling pathway. Therefore, CLC-3 might be a regulated target of TGF- β /TGF- β RII signaling pathway.

5. DISCUSSION

Diabetes is a serious metabolic disorder that can cause various system complications of the body, and a large number of studies have shown that the complications were mainly caused by high blood sugar: non-enzymatic saccharification process, redox stress, aldose reductase activation and two acylglycerol (DAG)/protein kinase C (PKC) pathway activation (24,25). The complications caused in the central nervous system is DE. Adult neurogenesis abnormalities were thought to be an important reason for cognitive disorder of DE. Hippocampal structure and the olfactory bulb were the most active regions for neurogenesis and the hippocampus neuron had degenerative change in the early diabetes (26), so high glucose had important role in the survival and growth of cells in the hippocampus (27-29).

The role of TGF- β and its receptor signaling pathways in some chronic complications of diabetes has been studied for a long time (30,31). The increased expression of TGF- β in the serum of diabetic patients with neuropathy was studied, which could

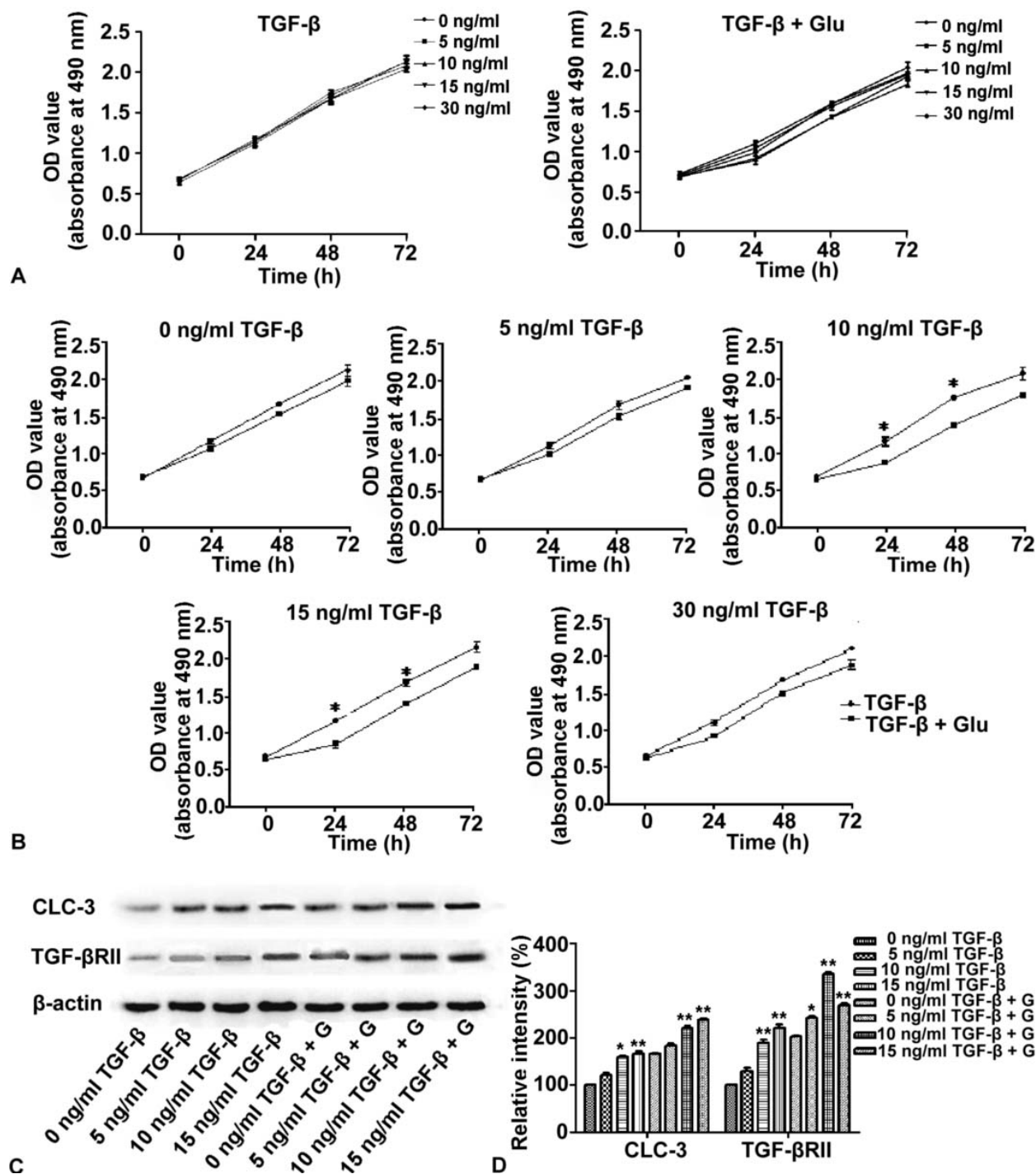


Figure 4. CCK-8 cell viability assay. The results were obtained from cells that were treated with different TGF-β for 24, 48 and 72 h with or without glucose. The expression of CLC-3 and TGF-βRII in cell models treated with TGF-β (25 mM) for 24 h were also recorded. (A) Evaluation of Cell proliferation under different concentrations of TGF-β with or without glucose. (B) The comparison of cell proliferation under condition after stimulation with different concentrations of TGF-β. (C) Representative bands of western blot and (D) densitometry analysis of CLC-3 and TGF-βRII under different concentrations of TGF-β. The TGF-β groups compared with 0ng/ml TGF-β group and the TGF-β + G groups compared with 0ng/ml TGF-β + G group, while G represents glucose. The statistical analysis was performed by ANOVA with post hoc Student-Newman-Keuls test. * $p < 0.05$; ** $p < 0.01$.

be as an indicator of diabetic peripheral neuropathy and had a negative correlation with nerve conduction (32). It showed that adult rats intraventricular infusion of TGF-β1 could reduce cell proliferation in the

hippocampal dentate gyrus and subventricular zone *in vivo* (33). It was found that the expression of TGF-β1 had related to cognitive disorder in the study of AD (34). Studies at home and abroad indicated that CLC-3 were

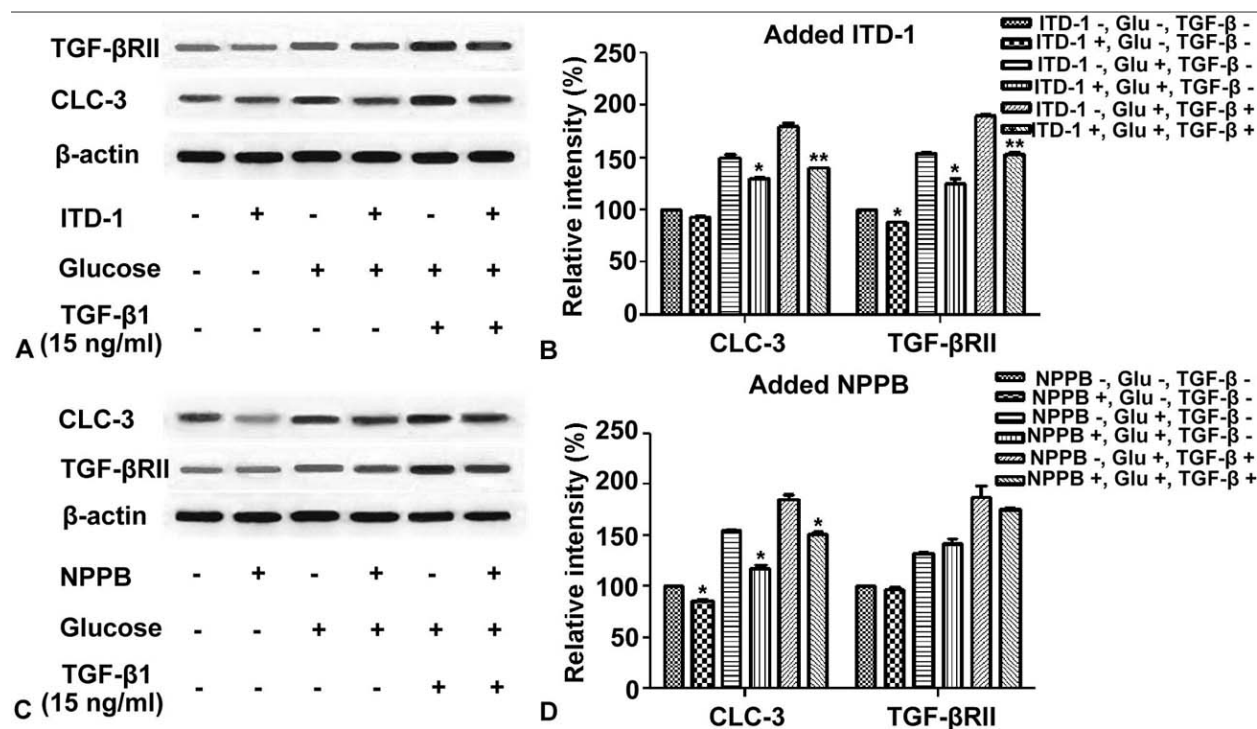


Figure 5. Western blot analysis on CLC-3 and TGF-βRII protein expressions treated with TGF-β inhibitor ITD-1 and CLC-3 inhibitor NPPB under high glucose condition in HT22 cells, respectively. (A) Representative bands of western blot and (B) densitometry analysis of CLC-3 and TGF-βRII under ITD-1 in the cells, data comparison between two adjacent groups. (C) Representative bands of western blot and (D) densitometry analysis of CLC-3 and TGF-βRII under NPPB in the cells. The statistical analysis was performed by ANOVA with post hoc Student-Newman-Keuls test. * $p < 0.05$; ** $p < 0.01$.

widely expressed in mammalian cells, mainly in the central nervous system, kidney and intestinal, which may be involved in the development of diabetes and its complications (35). The protein level of CLC-3 was specifically distributed, mainly expressed in the central nervous system and kidney. Stobrawa *et al.* reported that CLC-3 gene knockout mice almost congenital absence of the hippocampus and photoreceptors, and this may be related to long-term memory function (36). Our previous study also showed an increase in the expression of CLC-3 in neuronal HT22 cells at high glucose groups. Immunofluorescence method also found that CLC-3 was widely distributed in the cells, especially in cytoplasm, nucleus and cell membrane, and upregulated with high glucose stimulation.

Diabetes animal model, which was established mainly by the physical, chemical, biochemical or other pathogenic factors, was essential for the study of diabetes and its complications. Intraperitoneal injection of STZ was simple, short time-consuming and suitable for a large number of experiments, hence, in this study we applied STZ to induce the rats to be diabetes. In recent years, the nerve cells *in vitro* culture had become a commonly used method for studying the mechanism of neurons damage because it could rule out the error caused by many uncertain factors in the animal experimental procedure. Therefore, we chose high glucose to stimulate the HT22 cells to diabetes

state. Differentiated hippocampal neurons cells had better characteristics of hippocampal neuron model compared with no differentiated HT22 cells (37,38).

In this study, we also found that the expressions of CLC-3 and TGF-βRII were increased in the hippocampus of DM rats. CLC-3 was widely distributed in the neuronal cells and its expression in the hippocampus of diabetic rats was also increased by immunohistochemistry assay. The proliferation of HT22 cells in 25 mM glucose and TGF-β (10 and 15 ng/ml) stimulation groups was significantly lower than other groups at 24 h and 48 h. The expressions of CLC-3 and TGF-βRII in the HT22 cells under high glucose for 24 h was increased significantly when the TGF-β concentration was 10 ng/ml and 15 ng/ml. CLC-3 antagonists had no apparent influence on up-regulated TGF-βRII, but TGF-β1 inhibitors could reduce the up-regulated CLC-3 under high glucose. Therefore, the data suggested that TGF-β can stimulate the expression of CLC-3 under high glucose.

In DM, high glucose can induce changes in blood osmolarity, Cl-channel activity and TGF-β/TGF-βRII signaling pathway. In this study, CLC-3 and TGF-β signals might be related to cognitive disorder. The CLC-3 might be modulated by TGF-β/TGF-βRII signaling pathway to participate in the development

and progression of DE. Our previous research showed that CLC-3 was also expressed in the nucleus, so we suggest the TGF- β signal is activated, and then further activated CLC-3 in the nucleus. Taken together, CLC-3 might serve as a target of TGF- β /TGF- β RII signaling pathway and be involved in cognitive dysfunction and the pathogenesis of DE. And, the serum TGF- β 1 levels displayed a coordinated expression with TGF- β RII in DM rats.

In the present study, CLC-3 had high expression in the hippocampus of DM rats and the HT22 cells under high glucose, also cognitive disorders and low proliferation at the same time, and TGF- β /Smad signaling pathways could play a role in this process. These results demonstrated a role for the CLC-3 chloride channel in TGF- β /TGF- β RII signaling pathways, suggesting a novel target for the brain's nerve and cognitive capacity. It warrants further studies to investigate the possible change of TGF- β /TGF- β RII signaling pathway and CLC-3 in the response of long and short form variant of glucose and the diversity phase of DM. It might be meaningful to use serum TGF- β 1 level as an index for clinical diagnosis and treatment of DE. Serum TGF- β 1 may be a new molecular marker for diagnosing cognition degree and DE, also it warrants further studies to determinate the exact relationship between serum TGF- β 1 and cognitive impairment and its severity in diagnosed DE patients in real time. We hope the results of which could provide us with novel target molecules and mechanism, clinically aiding in slowing/preventing development of DE and other neurocognitive issues related to diabetes.

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Abbreviations: TGF- β : transforming growth factor beta; CLC-3: Chloride channel-3; TGF- β /TGF- β RII: transforming growth factor beta/transforming growth factor beta receptor; DM: diabetes mellitus; AD: alzheimer's disease; DE: diabetic encephalopathy; STZ: streptozotocin; FPG: fasting blood glucose; ELISA: enzyme-linked immunosorbent assay; FBS: fetal bovine serum; CCK-8: cell counting kit-8; OD: optical density; RIPA: radio immunoprecipitation assay; BCA: bicinchoninic acid; PVDF: polyvinylidene fluoride; TBST: tris-bufered saline with tween-20; ECL: electrochemiluminescence; DAB: 3-3-diaminobenzidine-4-HCL

Key Words: Diabetic rats, Learning, Memory, HT22, CLC-3, TGF-beta

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