

Original Article

Effects of Tianqijiangtang capsule on survival, self-renewal and differentiation of hippocampal neural stem cells of embryonic rats cultured in high glucose medium

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Abstract: Objective: This study aims to investigate the effects of Tianqijiangtang capsule on the survival, self-renewal and differentiation of hippocampal neural stem cells (NSCs) of embryonic rats cultured in high glucose medium. Materials and methods: A cell model of diabetic encephalopathy was established. Cell viability was assessed to screen the optimal concentration of glucose for the cell model of diabetic encephalopathy. Then, the effects of Tianqijiangtang capsule on the proliferation and differentiation of NSCs, and the expression of vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) in the culture medium and cells were detected. Results: High glucose significantly reduced the ability of survival, proliferation and differentiation of NSCs, which was statistically significant, when compared to the control group ($P < 0.05$ or 0.01). Tianqijiangtang capsule significantly enhanced the survival, proliferation and differentiation of NSCs cultured in high glucose medium, which was statistically significant, when compared with the high glucose group ($P < 0.05$ or 0.01). The high glucose culture resulted in a significant decrease in VEGF and BDNF levels in culture medium and cells of NSCs. Tianqijiangtang capsule significantly increased the level of VEGF nuclear BDNF in cells and the culture medium, which was significantly higher, when compared to that in the high glucose group ($P < 0.05$ or 0.01). Conclusion: Tianqijiangtang capsule enhances the level of neurotrophic factor synthesized and secreted by hippocampal NSCs cultured with high glucose through the autocrine and paracrine pathway, promotes the NSC survival, replication and differentiation of new neurons and astrocytes, and reduces the degeneration and necrosis of nerve cells.

Keywords: Tianqijiangtang capsule, diabetic encephalopathy, neural stem cells, vascular endothelial growth factor, brain-derived neurotrophic factor

Introduction

Diabetic complications, which are caused by diabetes and the state of glycaemia, involve the acute or chronic pathological change of the whole body, such as foot disease, kidney disease, eye disease, heart disease and encephalopathy, which are the most common complications of diabetes [1-3]. In particular, diabetic encephalopathy [4], which results in a large increase in the rate of disability and mortality of diabetes, and serious harm to the quality of life of patients, has increasingly become a hot

issue in society. At present, there is no effective drug to improve diabetic encephalopathy. Hence, seeking drugs or methods to prevent and cure diabetic encephalopathy have become a research hotspot.

As early as 1922, clinical research has suggested that diabetes can cause cognitive dysfunction [5], and De Jong RN [6] described this kind of cognitive disorder caused by diabetes as "diabetic encephalopathy" in 1950. At present, it has been generally considered that diabetic encephalopathy is a cognitive disorder caused

Effects of Tianqijiangtang capsule on neural stem cells

by diabetes mellitus and changes in the physiology and structure of the brain [7].

Diabetes-induced brain injury is the main clinical manifestation of cognitive impairment, including memory loss, decreased attention, decline of abstract thinking and reasoning ability, and visual dysfunction [8], which can eventually develop into dementia [9, 10]. From the perspective of various pathological mechanisms, the pathophysiology of diabetic patients may induce brain mechanisms, especially hippocampal synaptic information transmission disorder, neuron apoptosis, degeneration and death, leading to cognitive decline, and ultimately worsening as diabetic encephalopathy [11, 12].

Neural stem cells (NSCs) are cells that have the abilities of self-renewal, proliferation and differentiation, and these were found in monkey brains in the 90s of the last century [13]. NSCs can differentiate into neurons, astrocytes and oligodendrocytes, which constitute the brain tissue. These slow down aging, reduce the degeneration and death of cells, and maintain the structure and function of brain tissues [14]. NSCs are mainly located in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus in adult brains [15]. Furthermore, NSCs are normally in the resting state, and are activated when these receive nociceptive stimulation. Then, these proliferate and differentiate into new neurons, replace degenerated or necrotic nerve cells, and repair the structure and function of brains after injury [16-18].

Tianqijiangtang capsule is a commonly used drug for diabetes prevention and control in China at present, which lowers blood glucose levels in patients with diabetes [19]. Previous studies have indicated that Tianqijiangtang capsule controls diabetes by reducing hyperglycemia and modifying lipid metabolism [20], preventing diabetic vascular complications [21]. A clinical study revealed that Tianqijiangtang capsule can reduce blood glucose levels before meals and at two hours post-prandially in diabetic patients [22]. Furthermore, the conversion rate of impaired glucose tolerance developing as type II diabetes was 32.1%, and no toxic side effects were found [23].

The present study, which was designed on the basis of a previous study, isolated embryo rat

hippocampal neural stem cells, and investigated the effect and mechanism of action of Tianqijiangtang capsule on the survival, replication and differentiation of neurons and astrocytes of NSCs cultured by high glucose *in vitro*.

Materials and methods

Animal

Sprague-Dawley (SD) rats at gestation day 17 were provided by the Animal Institute, Peking Union Medical College (Certificate number: SCXK [Jing] 2016-0006). All procedures in the animal experiments followed the instructions for the care and use of animals provided by Beijing University of Chinese Medicine.

The Minimum Standards of Reporting Checklist contains the details of the experimental design, and the statistics and resources used in the present study.

Isolation, primary culture, passage culture and clone

Isolation and primary culture: Under sterile conditions and anesthesia, E17 embryonic rats were sterilized with 75% alcohol. The cranial cavity was opened, the meninges were peeled, the blood from the cortex was cleaned with anatomy liquid under an anatomical microscope, and the hippocampi were dissected in dissecting solution under a microscope. Then, the hippocampus was cut into 1-mm³ pieces in DMEM/F12 culture medium, and digested with 0.125% trypsin at 37°C for 25 minutes. The digestion was terminated by adding 10% fetal bovine serum. Afterwards, the digested hippocampal tissue was washed with DMEM/F12 to remove the serum, and was made into a single-cell suspension by trituration using a pipette. Next, the cell suspension was passed through a 200-mesh sieve. After cell counting (5×10^5 /mL), the harvested cells were grown in a constant temperature incubator (Thermo, China) at 37°C with 5% CO₂.

Passage culture: After culturing for 5-7 days, single cells that proliferated into the cell spheres were mechanically dissociated into the single cell suspension. Then, cell counting was performed under a microscope, and cell density was adjusted to 5×10^5 /ml and incubated in a 75-ml cell culture flask, once every 5-7 days.

Effects of Tianqijiangtang capsule on neural stem cells

Cloning: Next, the 5×10^5 /ml cell suspension was inoculated into a 96-well plate, and a single-cell hole was selected and marked under an inverted microscope, and cultured in a constant temperature incubator.

Tianqijiangtang capsule

A Tianqijiangtang capsule consists of 10 Chinese herbs: Astragali radix, Coptidisrhizoma, Trichosanthis radix, LigustriLucidifructus, Dendrobii caulis, Ginseng radix, Lycii cortex, Ecliptaetherba, Gallachinensis, and Cornifruetus. The quality of these herbs and decoction preparation was in accordance with the Chinese pharmacopoeia (2016). These were produced by Heilongjiang Baoquan Pharmaceutical Co. Ltd. (Hegang, Heilongjinag Province, China; Batch number: 150412).

Immunofluorescent staining

Part of the sub-cultured cells was taken and inoculated on coverslips coated with ploy-D-lysine in 24-well culture plates by differentiation culture medium.

BrdU labeling: BrdU was added into the cell culture medium at a final concentration of $10 \mu\text{g}/\text{ml}$. Then, these cells were cultured for four days until use for immunofluorescent staining. The harvested NSCs were cultured for four days and fixed with 4% paraformaldehyde. The groups and design of the fluorescent labeling experiment were carried out, as follows: Cells were plated onto coverslips coated with poly-D-lysine and immunofluorescent labeling for nestin/BrdU (to detect the self-renewal and proliferation of NSCs), BrdU/vimentin (to detect the astrocytic differentiation of NSCs) and BrdU/Tuj-1 (to detect the neuronal differentiation of NSCs) for 24 hours. The primary antibodies were nestin and BrdU (1:300 and 1:400, respectively), BrdU and vimentin (1:400 and 1:500, respectively), and BrdU and Tuj-1 (1:400 each). Goat anti-rabbit IgG-Cy3 (1:50) and goat anti-mouse IgG-FITC (1:50) were the secondary antibodies. Then, the counter staining of cell nuclei was carried out with DAPI (40, 6-diamidino-20-phenyindole, $100 \text{ ng}/\text{mL}$) for 10 minutes. All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Image-Pro Plus software was used to analyze the number, area density and optical density of positive cells in the fluorescence images.

Cell vitality assessment

Cells (5×10^4 /well) were plated into 96-well plates, and the MTT solution was prepared with 0.01 M of PBS (pH 7.4) at a concentration of 5 mg/ml. Then, 20 μL of MTT solution was added into each well and incubated for another four hours, the purple precipitates were dissolved with isopropanol with 0.04 M of HCl, and the optical density (OD) values were measured at a wavelength of 492 nm.

Enzyme-linked immunosorbent assay (ELISA)

The cell and cell-free supernatants were collected from treated and untreated NSCs in each group, and these were used to measure the production of VEGF and BDNF using an ELISA kit, according to manufacturer's protocol. The levels of VEGF and BDNF were determined and calculated with the result of the OD value at 450 nm.

Statistical analysis

All data were processed using SPSS 20.0. Data were expressed as mean \pm standard deviation (SD). The significance of variables was determined by one-way ANOVA and paired-sample *t*-test, with a statistical significance at the $P < 0.05$ level.

Results

Identification of hippocampal NSCs

Cells that were just dissected from fetuses in the suspension were single, small, and round or ellipse, with no processes. Cell clone spheres began to form at 24 hours after dissection. These were round after 3-4 days and passaged after 5-7 days (**Figure 1A-D**). The primary neurospheres had regular morphology, but had no irregularly sized processes, and the volume grew with culture time, which was the same as the passaged cell clone spheres. Single cells were cultured in a 96-well plate, and these single cells grew into cell spheres that comprised of dozens to hundreds of cells after 3-7 days (**Figure 1E-G**).

Cells were strongly nestin positive, as detected by the immunofluorescent staining. However, these nestin-positive cells varied in size, with the presence or absence of apophyses. Furthermore, positive red staining was observed in

Effects of Tianqijiangtang capsule on neural stem cells

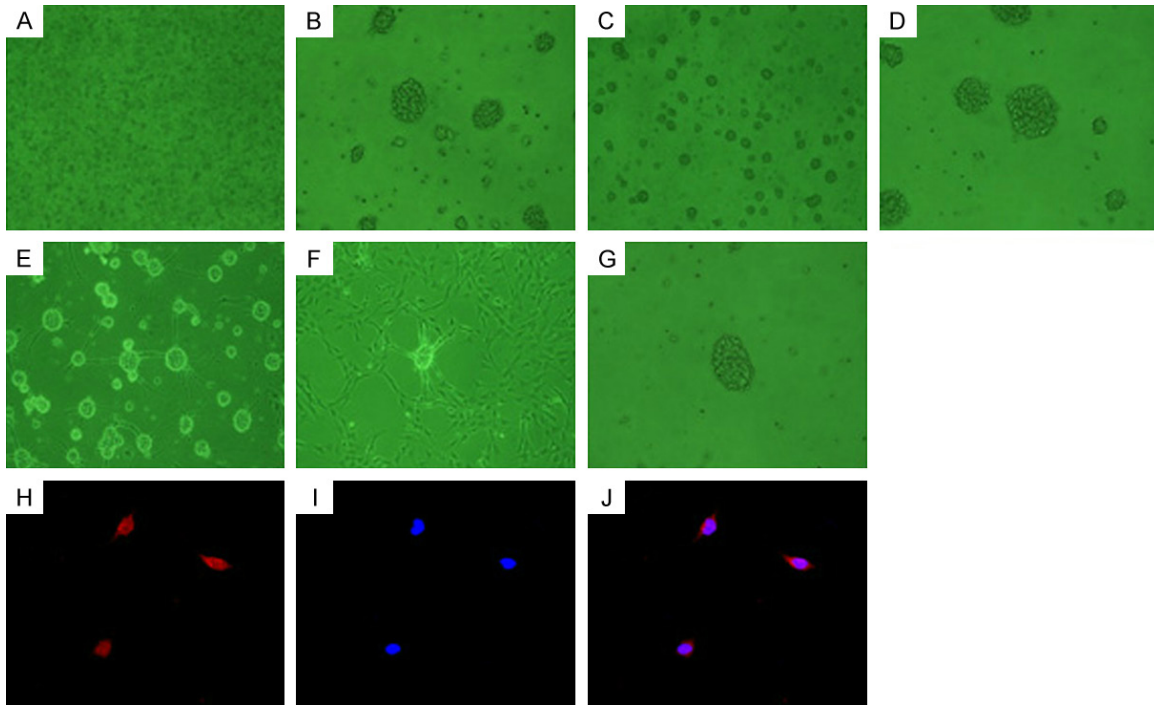


Figure 1. The culture of NSCs of embryonic rat hippocampus and Nestin positive cells of NSCs of embryonic rat hippocampus (600 \times). A: Primary culture for one day for NSCs obtained from embryonic rat hippocampus, 100 \times ; B: Primary culture for three days for NSCs obtained from embryonic rat hippocampus forming neurospheres, 200 \times ; C: Passage culture for one day for NSCs obtained from embryonic rat hippocampus, which formed neurospheres, 100 \times ; D: Passage culture for three days for NSCs obtained from embryonic rat hippocampus forming neurospheres, 200 \times ; E: Adherent culture for three days of passage for NSCs obtained from embryonic rat hippocampus, the edge of neurospheres appear with the processes, 100 \times ; F: Adherent culture for five days of passage for NSCs obtained from embryonic rat hippocampus, NSCs becoming differentiated, 100 \times ; G: Monoclonal culture for three days for NSCs obtained from embryonic rat hippocampus, single cell proliferation into neurospheres, 200 \times ; H: Nestin positive cells of NSCs obtained from embryonic rat hippocampus (red); I: DAPI nucleus of NSCs obtained from embryonic rat hippocampus (blue); J: The merge of nestin and DAPI for NSCs obtained from embryonic rat hippocampus.

the cytoplasm. These stained cells were round or oval-shaped, but their nuclear zones were not stained, and often displaced to one side of the cell (**Figure 1H-J**).

The positive dark-green staining in the nuclear area confirmed the expression of BrdU, with less staining in the cytoplasm. BrdU positive cells were small and had apophyses (**Figure 2A-D**).

A green color in the cytoplasm confirmed the expression of Tuj-1. The nuclear area of Tuj-1-positive cells was negative, and often displaced to one side of the cell. Tuj-1-positive cells were small and round, and had apophyses (**Figure 2E-H**).

Vimentin expression was observed with green staining in the cytoplasm. The vimentin-positive nuclear zones were negative, and often displaced to one side of the cell (**Figure 2I-L**).

Screening of optimal glucose concentration for the model of NSCs of embryonic rat hippocampus

A certain amount of glucose was added into DMEM-free medium, and the solution was filtered into concentrations of 4,000, 5,000, 6,000, 7,522, 8,500, 10,000, 15,088 and 20,000 $\mu\text{g/ml}$. Then, the MTT assay was performed to determine the optical density (OD) value, and this was compared with that in the control group. The best glucose concentration was 8,500 $\mu\text{g/ml}$. The results of the statistical analyses are presented in **Figure 3A**.

Screening of the optimal concentration and time point of Tianqijiangtang capsule in promoting the proliferation of neural stem cells

In the MTT assay, compared to the control group, the OD value markedly increased at doses of 5,000, 2,000, 1,000, 500, 200, 100, 50, 25,

Effects of Tianqijiangtang capsule on neural stem cells

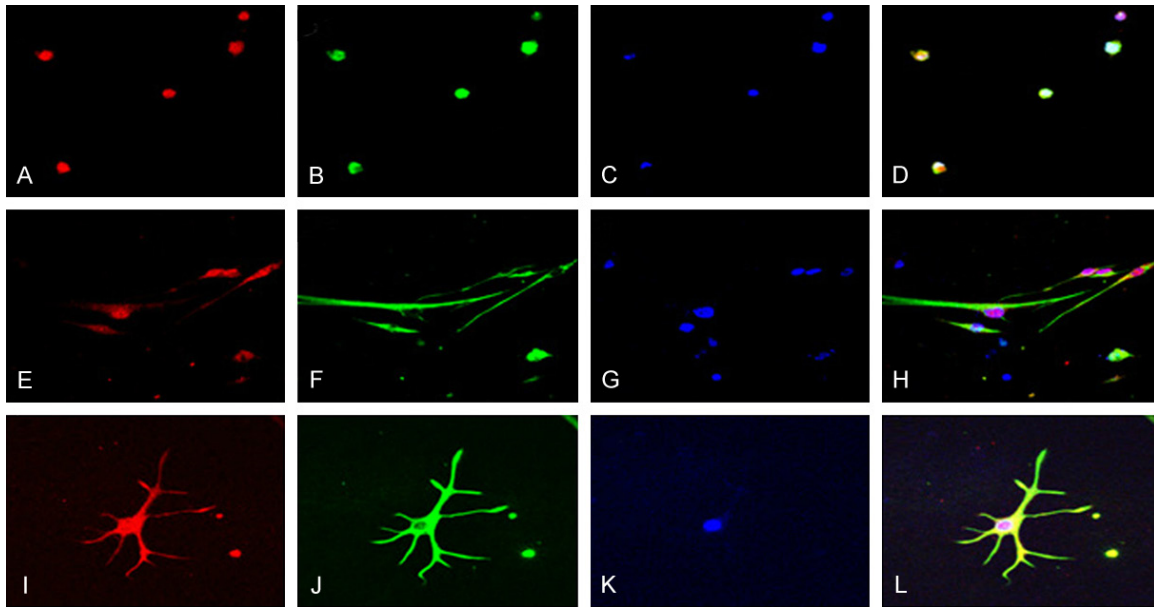


Figure 2. Nestin positive cells for NSCs obtained from embryonic rat hippocampus (A, red), BrdU positive cells for NSCs obtained from embryonic rat hippocampus (B, green), DAPI nucleus for NSCs obtained from embryonic rat hippocampus (C, blue), and the merge (D) of nestin, BrdU and DAPI for NSCs obtained from embryonic rat hippocampus (600 \times). Nestin positive cells for NSCs obtained from embryonic rat hippocampus (E, red), DAPI nucleus for NSCs obtained from embryonic rat hippocampus (F, blue), Tuj-1 positive cells for NSCs obtained from embryonic rat hippocampus (G, green), and the merge (H) of nestin, Tuj-1 and DAPI for NSCs obtained from embryonic rat hippocampus (600 \times). Nestin positive cells for NSCs obtained from embryonic rat hippocampus (I, red), vimentin positive cells for NSCs obtained from embryonic rat hippocampus (J, green), DAPI nucleus for NSCs obtained from embryonic rat hippocampus (K, blue), and the merge (L) of nestin, vimentin and DAPI for NSCs obtained from embryonic rat hippocampus (600 \times).

10, 5, 2.5, 1, 0.5, 0.25, 0.05, 0.01 and 0.005 $\mu\text{g}/\text{mL}$ of Tianqijiangtang capsule, with the highest value at 50 $\mu\text{g}/\text{ml}$. Hence, 50 $\mu\text{g}/\text{ml}$ was chosen for the remaining experiments. As the dose became higher than 100 $\mu\text{g}/\text{ml}$, the OD value decreased (**Figure 3B**).

With regard to the time of Tianqijiangtang capsule treatment at 50 $\mu\text{g}/\text{ml}$, the OD value in the Tianqijiangtang capsule group was not significantly different from that in the control group at 1-6 hours ($P > 0.05$). However, the OD value significantly increased at nine hours in the Tianqijiangtang capsule group, when compared to the control group ($P < 0.01$, **Figure 3C**). Therefore, for the present experiment, cells were pretreated with Tianqijiangtang capsule for nine hours.

Effect of Tianqijiangtang capsule on the proliferation and differentiation of neural stem cells obtained from embryonic rat hippocampus

Nestin was positively expressed in cells located in the cytoplasm. These cells were round or

oval, and mainly had two phenotypes: protuberant and non-protuberant. Furthermore, the nuclei were negative and occasionally partial to the cell side. Compared with the control group, the number of nestin positive cells decreased in the high glucose group, and these cells had no protrusions. Compared with the high glucose group, the number of positive cells significantly increased in the Tianqijiangtang capsule group.

BrdU staining was mainly found in the nucleus, and the cytoplasm also had a little staining. The number of BrdU positive cells decreased in the high glucose group, while the number of positive cells in the Tianqijiangtang capsule group was significantly higher than that in the high glucose group (**Figure 4**).

For the Tuj-1 and vimentin staining in the cytoplasm, the cell body was observed to have a small bulge, and the cell nucleus was negative and biased on the side of the cell. The number and processes of vimentin and Tuj-1 positive cells in the high glucose group obviously de-

Effects of Tianqijiangtang capsule on neural stem cells

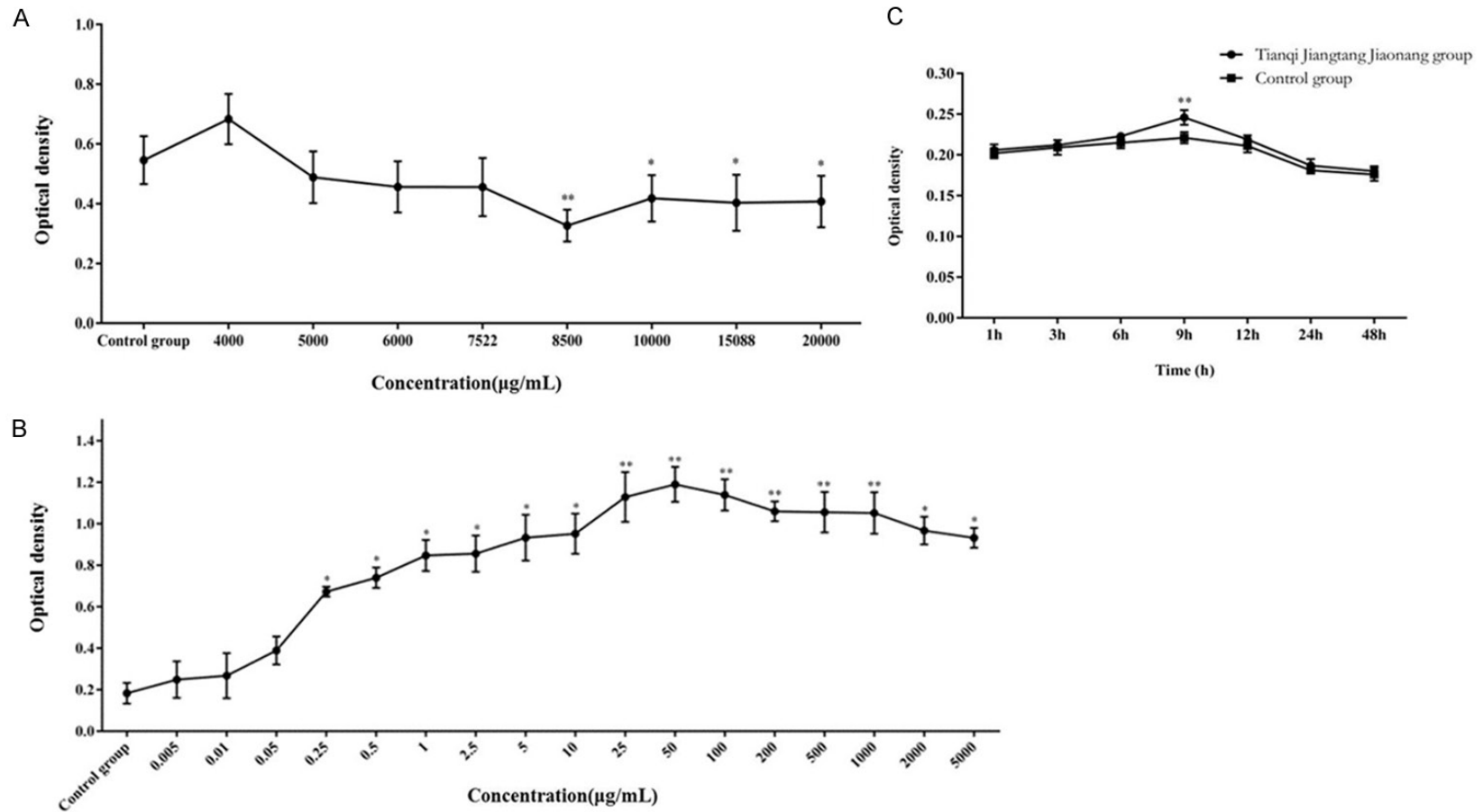


Figure 3. A. The screening of the optimal glucose concentration for the model of NSCs of embryonic rat hippocampus. The optimal concentration was 8,500 µg/ml. B. The screening of the optimal concentration of Tianqijiangtang capsule in promoting the proliferation of NSCs. The best concentration for the Tianqijiangtang capsule was 50 µg/ml. C. The screening of the best time point of 50 µg/ml of Tianqijiangtang capsule in promoting the proliferation of NSCs. The best time point was nine hours.

Effects of Tianqijiangtang capsule on neural stem cells

creased, when compared to that in the control group, and the cell volume also decreased. When compared to the high glucose group, the number of Tuj-1 and vimentin positive cells significantly increased in the Tianqijiangtang group, more processes were observed, and the cells clustered into groups (**Figures 5 and 6**).

The immunofluorescence analysis revealed that nestin, BrdU, Tuj-1 and vimentin were expressed in NSCs in the control group, high glucose group and Tianqijiangtang capsule group. Compared with the control group, the number of positive cells, optical density, area density and gray value were significantly lower in the high glucose group, and the difference was statistically significant ($P < 0.05$ or 0.01). Compared with the high glucose group, the number of positive cells, optical density and area density, and gray value were higher in the Tianqijiangtang capsule group, and the difference was statistically significant ($P < 0.05$ or 0.01).

Effect of Tianqijiangtang capsule on neurotrophic factors of rat embryonic neural stem cells cultured in high glucose

The ELISA method was used to detect the content of VEGF and BDNF protein in culture medium and cells. It was found that the VEGF and BDNF protein content of cells and culture medium in the high glucose group was significantly reduced, when compared with the control group, and the difference was statistically significant ($P < 0.05$ or 0.01).

Tianqijiangtang capsule significantly increased the content of VEGF and BDNF protein in culture medium and cells, when compared with the high glucose group, and the difference was statistically significant ($P < 0.05$ or 0.01 , **Figure 7**).

Discussion

The pathological lesion of the cognitive impairment in diabetic encephalopathy is damage of the hippocampus

The main clinical manifestation of diabetic brain damage is cognitive dysfunction, which include memory loss, decreased attention, decline in abstract thinking and reasoning ability, and visual dysfunction. Diabetic encephalopathy can develop dementia [4]. The total res-

ponse time and number of training errors increased in diabetic rats, which was induced by streptozotocin (STZ), and the latent period of the water maze was significantly longer than that in the normal group [24].

Studies have shown that for type-2 diabetes mellitus (T2DM) mice at 12 weeks, the ability of learning and memory decreased, the AChE activity in the cortex and hippocampus was enhanced, the expression of choline acetyltransferase in the hippocampus decreased, and the expression of neurotrophic factors and nerve growth factors was low. These results suggest that hyperglycemia in T2DM may lead to the degeneration of cholinergic neurons and the loss of cholinergic neurons in the hippocampus [25-27].

The T2DM mice model induced by the injection of STZ and combined with high fat diet has been shown to develop structural changes in hippocampal neurons and transduction pathway disorders in the insulin signal, which were mainly for the downregulation of insulin receptor neurons and the expression of the insulin receptor substrate in the hippocampus [28].

Studies have reported [27] that T2DM rat hippocampal pyramidal cells presented with obvious damage, including degeneration, necrosis, swelling, nuclear pyknosis, disorderly arranged organelles in cells, and CA1 and CA2 cells were significantly decreased in size and density, suggesting that the apoptosis of neural cells was involved in the pathogenesis of T2DM encephalopathy. Electrophysiological studies have shown that the synaptic plasticity of the hippocampus in T2DM rats decreased and the long-term potentiation was significantly inhibited [29, 30].

Effect of Tianqijiangtang capsule on the survival, reproduction and differentiation of neural stem cells in the hippocampus

The hippocampus is one of the main regions of neural stem cells in adult brains. Neural stem cells mainly exist in the subgranular zone of the dentate gyrus, which proliferates and migrate to the granule cell layer, differentiates into granule cells, slows down the aging, apoptosis and death of nerve cells, and maintains the structure and function of learning and memory of the dentate gyrus [31].

Effects of Tianqijiangtang capsule on neural stem cells

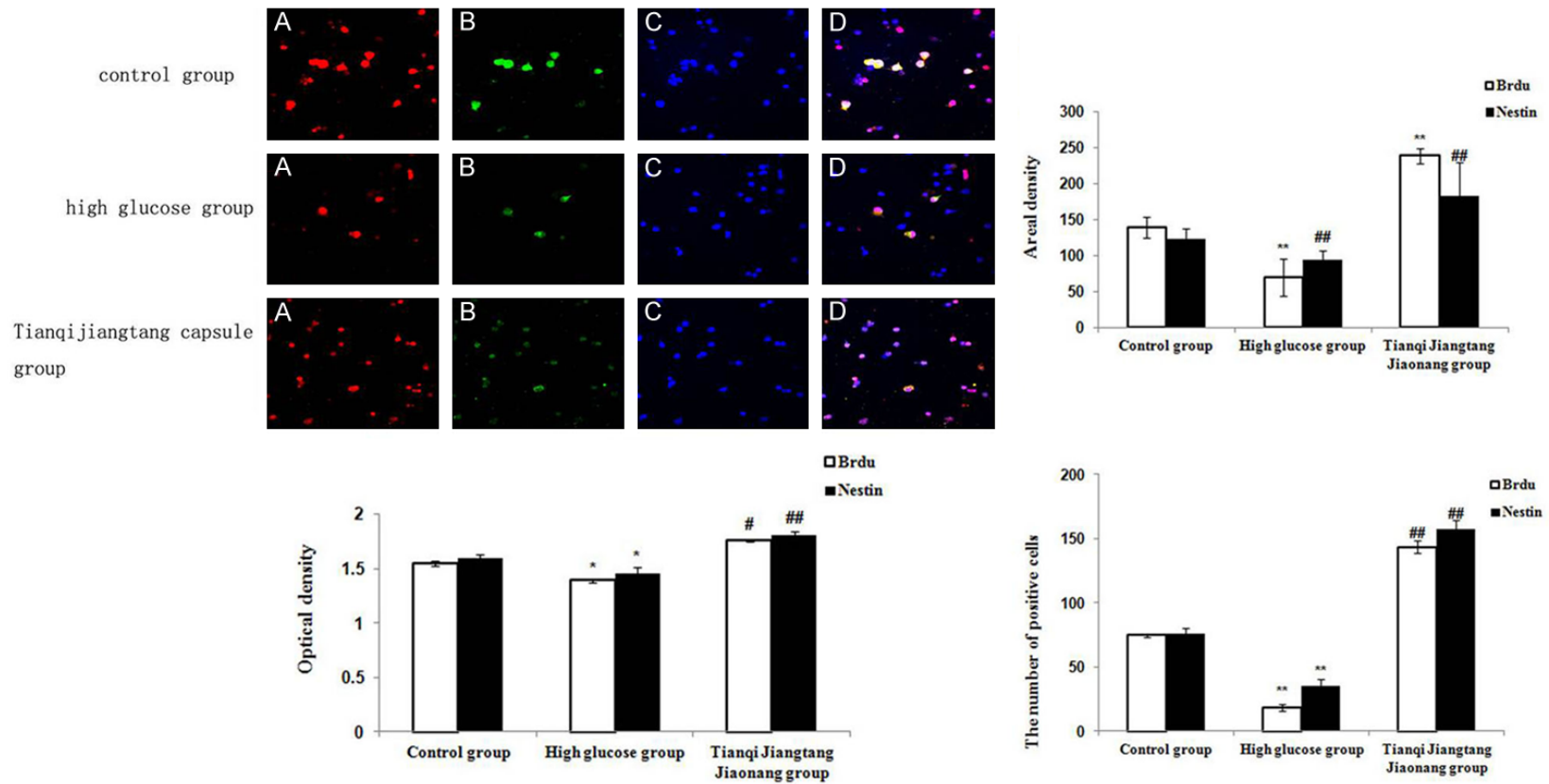


Figure 4. In control group, BrdU positive cells for NSCs obtained from embryonic rat hippocampus (A, red), Nestin positive cells for NSCs obtained from embryonic rat hippocampus (B, green), DAPI nucleus for NSCs obtained from embryonic rat hippocampus (C, blue), and the merge (D) of BrdU, nestin and DAPI for NSCs obtained from embryonic rat hippocampus (600 \times). In the high glucose group, BrdU positive cells for NSCs obtained from embryonic rat hippocampus (A, red), Nestin positive cells for NSCs obtained from embryonic rat hippocampus (B, green), DAPI nucleus for NSCs obtained from embryonic rat hippocampus (C, blue), and the merge (D) of BrdU, nestin and DAPI for NSCs obtained from embryonic rat hippocampus (600 \times). In the Tianqijiangtang capsule group, BrdU positive cells for NSCs obtained from embryonic rat hippocampus (A, red), Nestin positive cells for NSCs obtained from embryonic rat hippocampus (B, green), DAPI nucleus for NSCs obtained from embryonic rat hippocampus (C, blue), and the merge (D) of BrdU, nestin and DAPI for NSCs obtained from embryonic rat hippocampus (600 \times). Statistical results of the area and optical density, and the number of nestin, BrdU positive cells in the control, high glucose and Tianqijiangtang capsule group. Compared with the control group, * $P < 0.05$, ** $P < 0.01$; compared with the model group: # $P < 0.05$, ## $P < 0.01$.

Effects of Tianqijiangtang capsule on neural stem cells

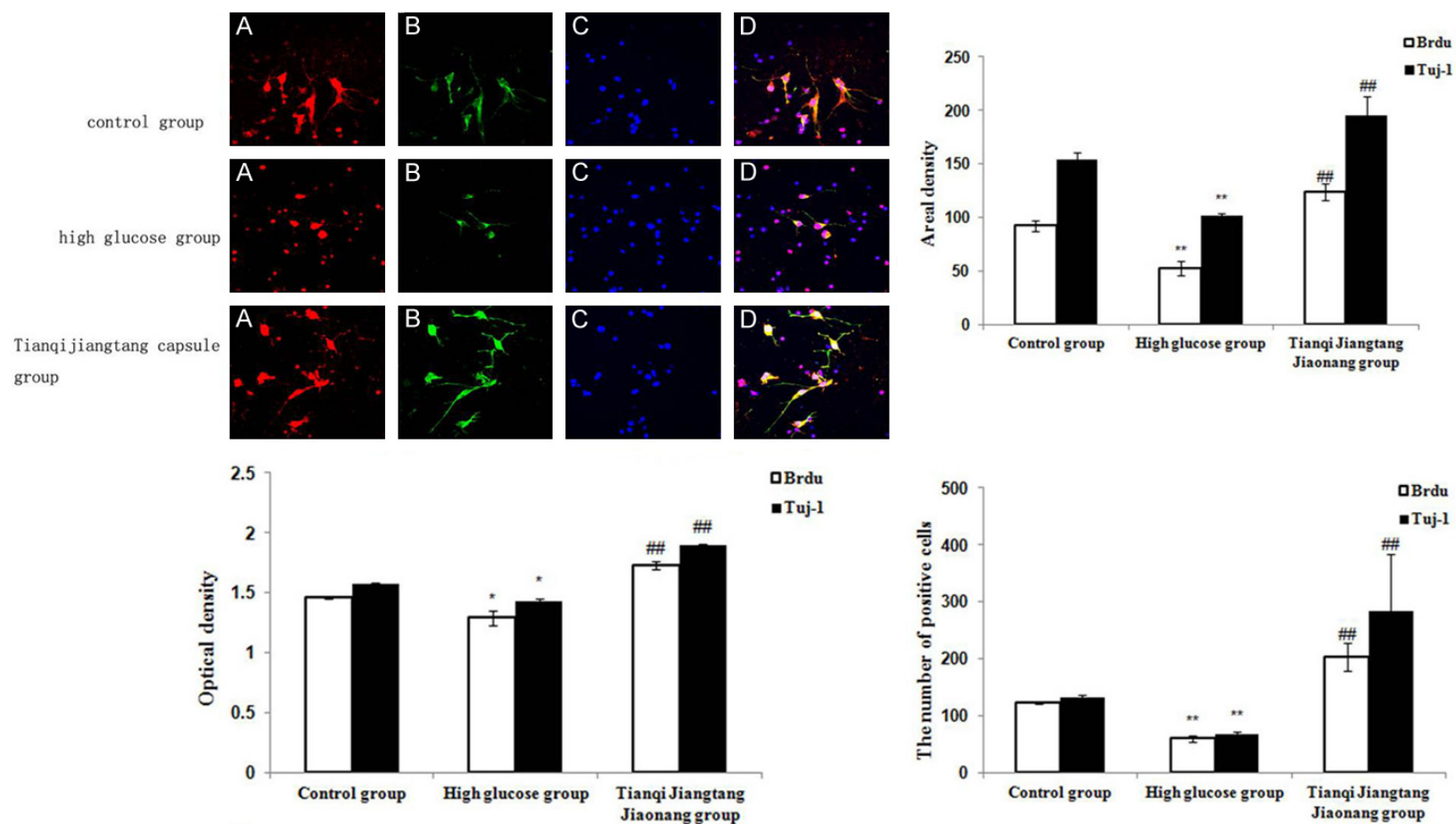


Figure 5. In the control group, BrdU positive cells for NSCs obtained from embryonic rat hippocampus (A, red), Tuj-1 positive cells for NSCs obtained from embryonic rat hippocampus (B, green), DAPI nucleus for NSCs obtained from embryonic rat hippocampus (C, blue), and the merge (D) of BrdU, Tuj-1 and DAPI for NSCs obtained from embryonic rat hippocampus (600 \times). In the high glucose group, BrdU positive cells for NSCs obtained from embryonic rat hippocampus (A, red), Tuj-1 positive cells for NSCs obtained from embryonic rat hippocampus (B, green), DAPI nucleus for NSCs obtained from embryonic rat hippocampus (C, blue), and the merge (D) of BrdU, Tuj-1 and DAPI for NSCs obtained from embryonic rat hippocampus (600 \times). In the Tianqijiangtang capsule group, BrdU positive cells for NSCs obtained from embryonic rat hippocampus (A, red), Tuj-1 positive cells for NSCs obtained from embryonic rat hippocampus (B, green), DAPI nucleus for NSCs obtained from embryonic rat hippocampus (C, blue), and the merge (D) of BrdU, Tuj-1 and DAPI for NSCs obtained from embryonic rat hippocampus (600 \times). The results of the statistical analyses of the area and optical density, and number of Tuj-1 and BrdU positive cells in the control, high glucose and Tianqijiangtang capsule group. Compared with the control group, * $P < 0.05$, ** $P < 0.01$; compared with the model group, # $P < 0.05$, ## $P < 0.01$.

Effects of Tianqijiangtang capsule on neural stem cells

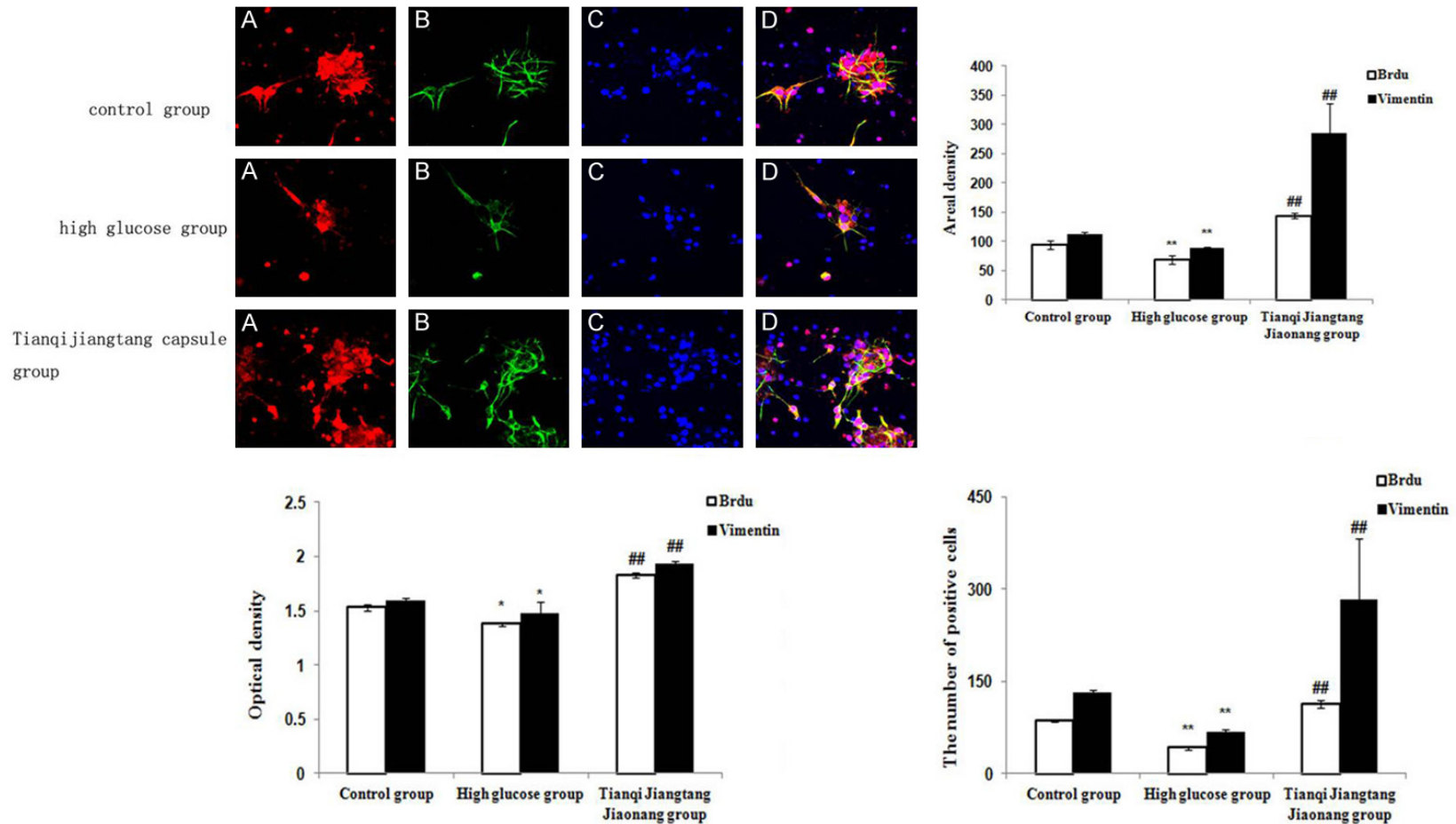


Figure 6. In the control group, BrdU positive cells for NSCs obtained from embryonic rat hippocampus (red), vimentin positive cells for NSCs obtained from embryonic rat hippocampus (green), DAPI nucleus for NSCs obtained from embryonic rat hippocampus (blue), and the merge of BrdU, vimentin and DAPI for NSCs obtained from embryonic rat hippocampus (600 \times). In the high glucose group, BrdU positive cells for NSCs obtained from embryonic rat hippocampus (red), vimentin positive cells for NSCs obtained from embryonic rat hippocampus (green), DAPI nucleus for NSCs obtained from embryonic rat hippocampus (blue), and the merge of BrdU, vimentin and DAPI for NSCs obtained from embryonic rat hippocampus (600 \times). In the Tianqijiangtang capsule group, BrdU positive cells for NSCs obtained from embryonic rat hippocampus (red), vimentin positive cells for NSCs obtained from embryonic rat hippocampus (green), DAPI nucleus for NSCs obtained from embryonic rat hippocampus (blue), and the merge of BrdU, vimentin and DAPI for NSCs obtained from embryonic rat hippocampus (600 \times). Statistical results of the area and optical density, and number of vimentin and BrdU positive cells in the control, high glucose and Tianqijiangtang capsule group. Compared with the control group: * $P < 0.05$, ** $P < 0.01$; Compared with the model group, # $P < 0.05$, ## $P < 0.01$.

Effects of Tianqijiangtang capsule on neural stem cells

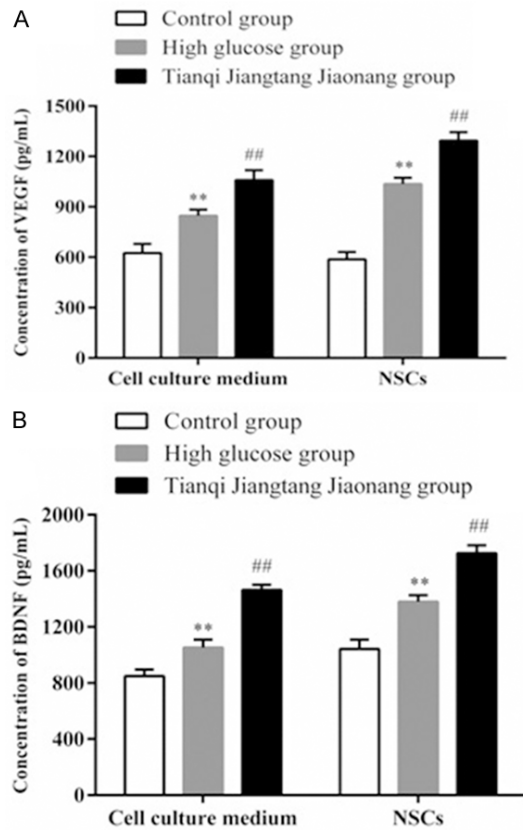


Figure 7. A. The results of the statistical analyses of the content of VEGF and BDNF in the culture medium. Compared with the control group: * $P < 0.05$, ** $P < 0.01$; Compared with the model group: # $P < 0.05$, ## $P < 0.01$. B. The results of the statistical analyses of the content of VEGF and BDNF in cells. Compared with the control group, * $P < 0.05$, ** $P < 0.01$; Compared with the model group: # $P < 0.05$, ## $P < 0.01$.

In the present study, rat embryonic hippocampal neural stem cells were isolated and subjected to primary culture and passaged, and the single cell was cloned and cultured. Then, cells were identified by nestin/DAPI immunofluorescence staining. These cultured cells were neural stem cells, and the purification rate was 98%. At the same time, with the use of immunofluorescence staining for BrdU/nestin/DAPI, Tuj-1/BrdU/DAPI and vimentin/BrdU/DAPI, it was confirmed that cultured cells have the biological characteristics of NSCs, which can self-replicate and differentiate into neuronal precursor cells and astrocyte precursors.

In the present study, the MTT method was used to screen out the optimal glucose concentration (8,500 $\mu\text{g/ml}$), simulating the hippocampal damage of diabetic encephalopathy induced by

hyperglycemia. The MTT assay revealed that the optimal dosage of Tianqijiangtang capsule is 50 $\mu\text{g/ml}$, and the best time-window is nine hours.

The ability of NSCs to proliferate was decreased by the high glucose culture. Compared with the control group, the number, area density and optical density of BrdU and nestin positive cells significantly decreased ($P < 0.05$ or 0.01). Furthermore, compared with the high glucose group, the Tianqijiangtang capsule can significantly increase the number, area density and optical density of BrdU and nestin positive cells ($P < 0.05$ or 0.01). These results reveal that the Tianqijiangtang capsule can promote the survival and replication of NSCs under high glucose injury.

The ability of neural stem cells to differentiate into neurons and astrocytes was decreased by the high glucose culture. Compared with the control group, the number, area density and optical density of Tuj-1 and vimentin positive cells significantly decreased ($P < 0.05$ or 0.01). Furthermore, compared with the high glucose group, Tianqijiangtang capsule can significantly increase the number, area density and optical density of Tuj-1 and vimentin positive cells ($P < 0.05$ or 0.01). These results show that the Tianqijiangtang capsule could promote the differentiation of neural stem cells into neurons and astrocytes under high glucose injury.

The high glucose culture resulted in the significant decrease in levels of VEGF and BDNF in the culture medium and cells of NSCs. Compared with the control group, the difference was statistically significant ($P < 0.05$ or 0.01). Furthermore, Tianqijiangtang capsule significantly increased the level of VEGF nuclear BDNF in the cell and culture medium, which was significantly higher than that in the high glucose group ($P < 0.05$ or 0.01). These results revealed that the Tianqijiangtang capsule has the effect of promoting the synthesis and secretion of neurotrophic factors in neural stem cells under high glucose damage.

Conclusion

Tianqijiangtang capsule enhanced the level of neurotrophic factor synthesized and secreted by hippocampal NSCs, and cultured with high glucose through the autocrine and paracrine

pathway, promoting NSC survival, and the replication and differentiation of new neurons and astrocytes, replacing the degeneration and necrosis of nerve cells, and improving the cognitive impairment of diabetic encephalopathy.

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Disclosure of conflict of interest

None.

Abbreviations

NSCs, neural stem cells; MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium, bromide, Thiazolyl Blue Tetrazolium Bromide; VEGF, vascular endothelial growth factor; BDNF, brain-derived neurotrophic factor; SVZ, subventricular zone; SGZ, subgranular zone; cFDA, the state food and drug administration of China; OD, optical density; STZ, streptozotocin.

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