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Effect of thioredoxin-interacting protein on Wnt/β-catenin signaling pathway and diabetic myocardial infarction

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ABSTRACT

Objective: To explore the regulatory role of thioredoxin-interacting protein (TXNIP) in Wnt/β-catenin signaling pathway and therefore to elucidate its function in diabetic myocardial infarction.
Methods: Diabetic myocardial infarction models were generated in mice. The expression levels of TXNIP and β-catenin and level of reactive oxygen species (ROS) were determined and compared with those in control group. Human umbilical vein endothelial cells were treated with high-concentration glucose and/or silencing TXNIP and/or H_2O_2. After 24 h, expression levels of TXNIP, β-catenin and its downstream protein Cyclin D1, and *C-myc* gene were determined by real-time PCR, Western blot and immunofluorescence method. The cell proliferation and ROS production capability in different groups were determined by methyl thiazolyl tetrazolium assay.
Results: Compared with control group, hyperglycemia significantly up-regulated TXNIP expression and ROS level in the myocardium and endothelial cells of myocardial infarction area, whereas the β-catenin expression was down-regulated, and the difference was statistically significant ($P < 0.05$). In comparison with Human umbilical vein endothelial cells in the control group, high glucose level increased the levels of TXNIP expression and ROS level in cells, but reduced cell proliferation as well as migration capability and expression levels of β-catenin, Cyclin D1 and C-myc; the difference was statistically significant ($P < 0.05$). However, this trend can be partially reversed by silencing TXNIP.
Conclusions: Diabetic myocardial ischemia could up-regulate levels of TXNIP expression and ROS production in endothelial cells of myocardial infarction area. The regulation effect of TXNIP on β-catenin was partially achieved by changing ROS levels.

1. Introduction

At present, there are more than 300 million diabetes patients all around the world and the number would reach near 400 million till 2025 [1]; in addition, more than 400 million people are at stake of prediabetic state [2]. In China, there are near 90 million diabetes patients [3], nearly accounting for 1/3 of the globe. Obviously, diabetes has become the major factor for influencing the people's health in both China and around the world [4,5]. Researches show that diabetes can raise the morbidity of cardiovascular and cerebrovascular diseases in patients [6], and increase the occurrence rate of myocardial infarction, heart failure, death and others [7]. Therefore, it is of great significance to study the action mechanism of diabetes in myocardial infarction and other diseases, as well as the significance of treatment in improving the patients' health state and decreasing the incidence rate of cardiovascular diseases.

The formation of coronary collateral circulation and angiogenesis is a kind of compensatory protection mechanism secondary to myocardial ischemia. Meier *et al* found that branching

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of coronary collaterality could decrease the 36% mortality risk in patients with coronary artery disease [8]. However, diabetes has an inhibitory effect on the formation of coronary collateral circulation [9,10], which may be the important reason for the high incidence rate of cardiovascular disease and other illness in diabetes patients. In the other hand, the scarce study on the relative internal mechanism is not good for the definition of pathogenesis of diabetes as well as the treatments for it. Researches show that Wnt/β-catenin signaling pathway is of significance in cell proliferation, angiogenesis, cardial development and repair, *etc* [11–13]. Thioredoxin-interacting protein (TXNIP) is a sort of protein playing an important role in the development of diabetes, whose expression can be regulated by the blood glucose levels [14]. Nevertheless, there are no relevant reports about what kind of connection exists between TXNIP and Wnt/β-catenin signaling pathway, and how they play a regulatory role in diabetic myocardial infarction. The present study generated the diabetic myocardial infarction models in rats for the analysis of changes in TXNIP, as well as β-catenin and its downstream proteins Cyclin D1, and human umbilical vein endothelial cells (HUVEC) for the analysis of changes in c-Myc and reactive oxygen species (ROS) after high concentrations of glucose treatment, respectively, to explore the action mechanism of TXNIP and Wnt/β-catenin signaling pathway as well as their roles in diabetic myocardial infarction, so as to provide the scientific reference for treating diabetic myocardial infarction through TXNIP.

2. Materials and methods

2.1. Experimental materials

Male C57BL/6 rats aged 8 wk and weighed 20 g were purchased from Shanghai WuXi AppTec Incorporation and raised and domesticated in the experimental conditions for a week before the experiment. The temperature and circadian rhythms and other factors were maintained constant, and good ventilation was achieved during the period of domestication.

HUVEC was purchased from Nanjing KeyGEN Biotech. Co., LTD and raised in an incubator with 5% CO_2 and dulbecco's modified eagle medium at 37 °C.

2.2. Experiment design

2.2.1. Animal handling

Rats with the same age and similar weights were starved for 12 h. Some of them were treated with intraperitoneal injection of 50 mg/kg streplozotocin to generate the diabetes models. If higher than 11 mmol/L blood glucose of was observed for continuous 3 d, then it meant the modeling was successful, and the rats were continued to be raised for a month.

The rats which were not generated into diabetes models and the diabetes models which were raised after a month were treated as follows respectively:

I: Non-diabetes + sham operation (NS); II: Non-diabetes + myocardial infarction (NMI); III: Diabetes + sham operation (DS); IV: Diabetes + myocardial infarction (DMI).

The rats with myocardial infarction were fixed on the laboratory table after 2% isoflurane anesthesia, and the thoracic cavity was cut off carefully. The left anterior descending coronary artery was ligatured at the inferior position of left auricle with 6/0 no damage suture line, and then the heart was put back and the chest was closed. The rats treated with sham operation were in the same situation without the ligatured left anterior descending coronary artery though.

After the treatment, the rats in each group were raised for 4 d until their hearts were taken out to clip the myocardial infarction tissue for determination of the expression level of TXNIP, ROS production and other indicators.

2.2.2. Cell handling

After the HUVEC was cultured to the fourth generation, cells at logarithmic phase were digested with 0.25% pancreatic enzyme and inoculated in a 6-well plate. Then, 33 mmol/L glucose or culture of 33 mmol/L glucose + [H_2O_2 and/or silencing TXNIP (siTXNIP)] was added as follows:

a) 5.5 mmol/L glucose (control); b) 33 mmol/L glucose; c) 33 mmol/L glucose + siTXNIP; d) 33 mmol/L glucose + H_2O_2; e) 33 mmol/L glucose + H_2O_2 + siTXNIP.

For each handling, 5 repetitive wells were established and 24 h after the handling, the culture was abandoned. TXNIP and β-catenin expression levels in cells, ROS production and other indicators were determined.

2.3. Determination of experimental indicators

2.3.1. Determination of protein expression in heart tissue and HUVEC

The expression levels of TXNIP and β-catenin in heart tissue and HUVEC were determined by Western blot. After the experiment, myocardial infarction tissue or handled cells in each group was taken, and radio-immunoprecipitation assay lysis buffer was added and then the mixture was lay on the ice to undergo lysis for 30 min. After 10-min centrifugation at 4 °C and 12 000 r/min, the supernatant was collected. Through sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins in samples were separated and transferred to polyvinylidene difluoride. The 5% skimmed milk powder was used to seal polyvinylidene difluoride and then corresponding primary antibody was added and kept at 4 °C overnight before tris buffered saline tween was used to wash the membrane. Then, the corresponding second antibody was added (1:3 000) and incubated at room temperature for 1 h. After the second membrane washing with tris buffered saline tween, ECL-plus luminescence liquid was used for rendering color. The relative expression was expressed by the specific value of gray levels of protein bands and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) bands.

2.3.2. Determination of fluorescence levels of TXNIP in heart tissue and HUVEC

After the experiment, 5 rats in each group were dissected. The hearts were OCT-embedded and frozen with liquid nitrogen, after which frozen sections were cut and kept at −20 °C. For HUVEC, cells were collected; and after culture medium was

abandoned, cells were fixed with 4% paraformaldehyde for 10 min and then washed.

Upon determination, the frozen sections were taken out and rewarmed at temperature for 30 min. Then, 3‰ goat serum was added into frozen sections, or HUVEC that was frozen with paraformaldehyde for sealing treatment for 1 h. Afterwards, mixed primary antibody [rat CD31 monoclonal antibody (endothelial cell marker) and rat TXNIP monoclonal antibody] was added and kept at 4 °C overnight. After being rewarmed for 30 min, phosphate buffer (PBS) was used to wash them and mixed second antibody (goat anti-rat Flour-594 fluorescence second antibody and goat anti-rat 488 fluorescence second antibody) was added for sealing treatment for 1.5 h. After second washing with PBS, 4′,6-diamidino-2-phenylindole was added. PBS was used to wash them again and 50% glycerinum was used to seal the sections, after incubation at 37 °C for 10 min. Then, laser scanning confocal microscope (Nikon, Japan) was used to determine the fluorescence level of TXNIP in endothelial cells of myocardial infarction area. A total of 5 parts of each rat were shot and the TXNIP amount was calculated.

2.3.3. Determination of cell proliferation and migration capability of HUVEC

Cells at logarithmic phase were taken to culture with 5% CO_2 at 37 °C for 24 h. With reference to the handling in Part 2.2.2., corresponding fresh culture (density of 2×10^4/well) was used instead and the cells were cultured for 24, 48, 72 and 96 h, after which methyl thiazolyl tetrazolium colorimetry assay was used to determine the cytoactivity and cellular proliferation inhibition rate was calculated with 5 repetitive wells established. Upon determination, methyl thiazolyl tetrazolium (5 g/L) 20 μL was added to each well and the incubation was continued for 4 h before the culture solution was abandoned. Then, 15 μL dimethyl sulfoxide was added and the mixture was shaken for 10 min after which the absorbance was determined at 490 nm.

2.3.4. Determination of TXNIP, Cyclin D1 and C-myc mRNA levels in HUVEC

The real-time PCR was used to quantify the levels of *TXNIP*, *Cyclin D1* and mRNA of *C-myc* in HUVEC. Upon determination, a moderate amount of cell samples were taken and Trizol reagent was added. According to the RNA extraction kit (Invitrogen, USA) protocol, the total RNA was extracted and the extracted RNA amount (A_{260}) as well as purity (A_{260}/A_{280}) was determined. A total of 1 μg total RNA was taken to reversely transcript to the first strand of cDNA, after which the according primers were added for amplification with *GAPDH* as the reference gene (Table 1). The results of amplification were analyzed by using $2^{-\Delta\Delta Ct}$ method.

2.3.5. Determination of ROS

With reference to the method in Part 2.3.2., after the tissue sections and collected HUVEC were sealed with goat serum, the ROS fluorescence probe—dihydroethidium, was added and incubated at 37 °C for 1.5 h. After PBS washing and the addition of 4′,6-diamidino-2-phenylindole, the ROS levels in heart and

cells were determined by using laser scanning confocal microscope based on the previous method.

2.4. Statistical analysis

Data were expressed as mean ± SD. One-way ANOVA was conducted by using SPSS20.0 and independent sample *t* was used to determine the difference between groups. Results with $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Effect of high glucose on TXNIP in heart tissue

Figure 1 showed that compared with control group, the expression levels of TXNIP mRNA and proteins in rats' myocardium in DS group were significantly increased, and TXNIP expression levels in endothelial cells of heart increased, with statistically significant difference ($P < 0.05$). Compared with NMI group, the expression levels of TXNIP in myocardial infarction area and endothelial cells of heart in DMI group were significantly increased, with statistically significant difference ($P < 0.05$).

3.2. Effect of high glucose on TXNIP expression in HUVEC

The results of PCR, Western blot and immunofluorescence method showed in Figure 2 that 24 h after high glucose treatment, TXNIP expression in HUVEC cells in HG group was significantly higher than that in control group, with statistically significant difference ($P < 0.05$). Compared with HG group, TXNIP expression in siTXNIP + HG group significantly decreased, with statistically significant difference ($P < 0.05$).

3.3. Effect of high glucose on cell proliferation and migration capability of HUVEC

Results of methyl thiazolyl tetrazolium assay showed that cell proliferation of HUVEC in HG group was significantly lower than that in control and siTXNIP + HG groups, with statistically significant difference ($P < 0.05$) (Figure 3A). Figure 3B showed that cell migration capability in HG group was significantly lower than that in control and

Table 1

The primers sequence in this experiment.

Primers		Sequence
TXNIP	F	5′-CAGAAGCTCCTCCCTGCTATATG-3′
	R	5′-GATGCAGGGATCCACCTCAG-3′
Cyclin D1	F	5′-TCTACACCGACAACTCCATCC-3′
	R	5′-GTGTTTGCGGATGATCTGTTT-3′
C-myc	F	5′-CCTCCACTCGGAAGGACTATC-3′
	R	5′-TGTTCGCCTCTTGACATTCTC-3′
GAPDH	F	5′-GACCTGACCTGCCGTCTA-3′
	R	5′-AGGAGTGGGTGTCGCTGT-3′

siTXNIP + HG groups, with statistically significant difference ($P < 0.05$).

3.4. Effect of high glucose on ROS levels in heart and HUVEC

Compared with control group, ROS level in heart in DS group was significantly increased and meanwhile ROS level in DMI was also higher than that in NMI group, with statistically significant difference ($P < 0.05$) (Figure 4A). Compared with HUVEC in control group, the ROS level in HG was significantly increased and higher than that in siTXNIP + HG group, with statistically significant difference ($P < 0.05$) (Figure 4B).

3.5. Effect of high glucose on β-catenin in heart and β-catenin, Cyclin D1 and C-myc expression levels in HUVEC

Compared with control group, β-catenin expression level in heart in DS group was significantly decreased and meanwhile β-catenin expression level in DMI group was significantly lower than that in NMI group, with statistically significant difference ($P < 0.05$) (Figure 5A). Compared with HUVEC in control group, the expression levels of *β-catenin*, *Cyclin D1* and *C-myc* were significantly decreased in HG group, and all indicators in HG group were lower than those in siTXNIP + HG group, with statistically significant difference ($P < 0.05$).
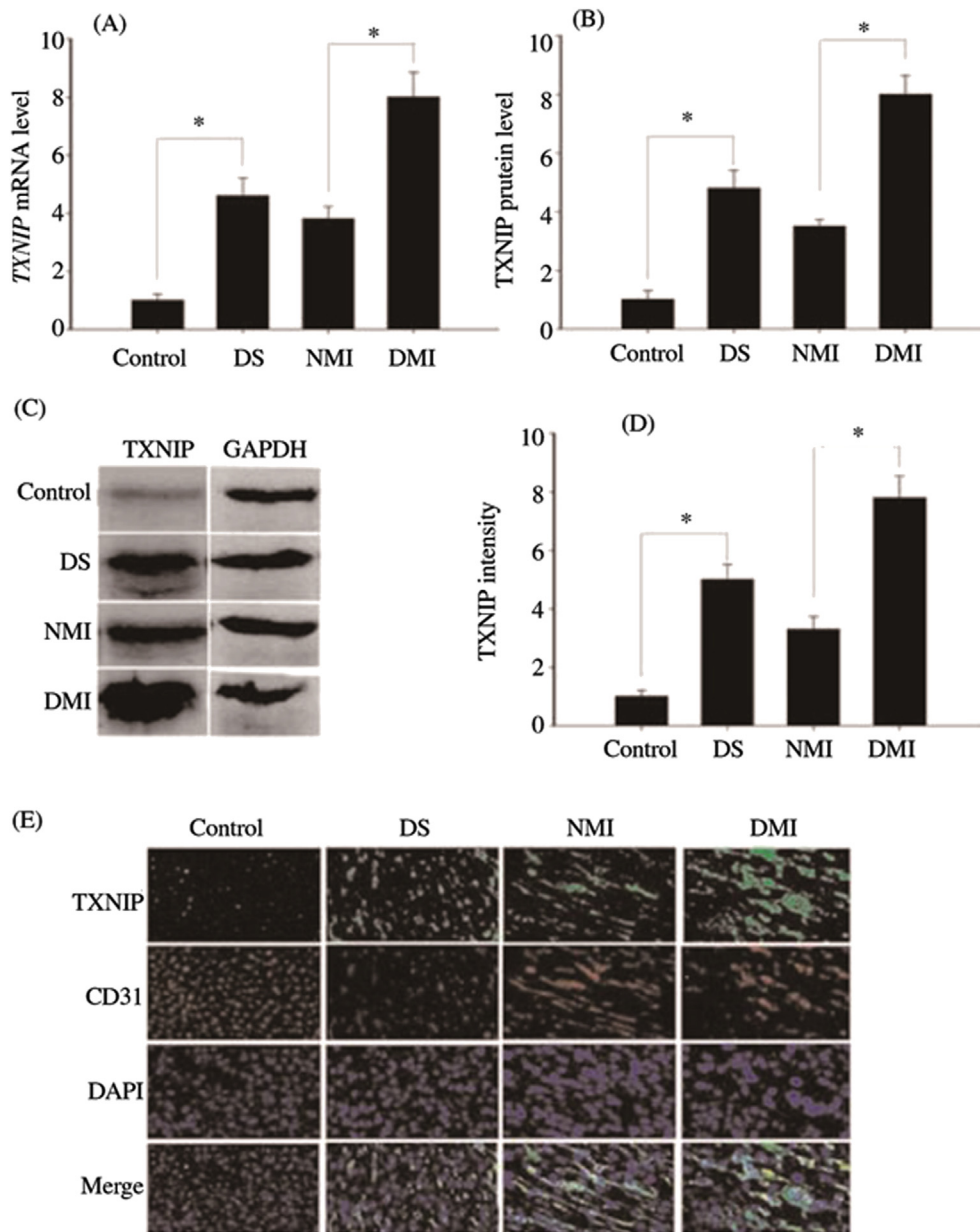

Figure 1. TXNIP expression levels in different groups.
A: *TXNIP* mRNA in myocardium; B, C: TXNIP proteins in myocardium; D, E: TXNIP in myocardial endothelial cells; *: Statistically significant difference between groups ($P < 0.05$).
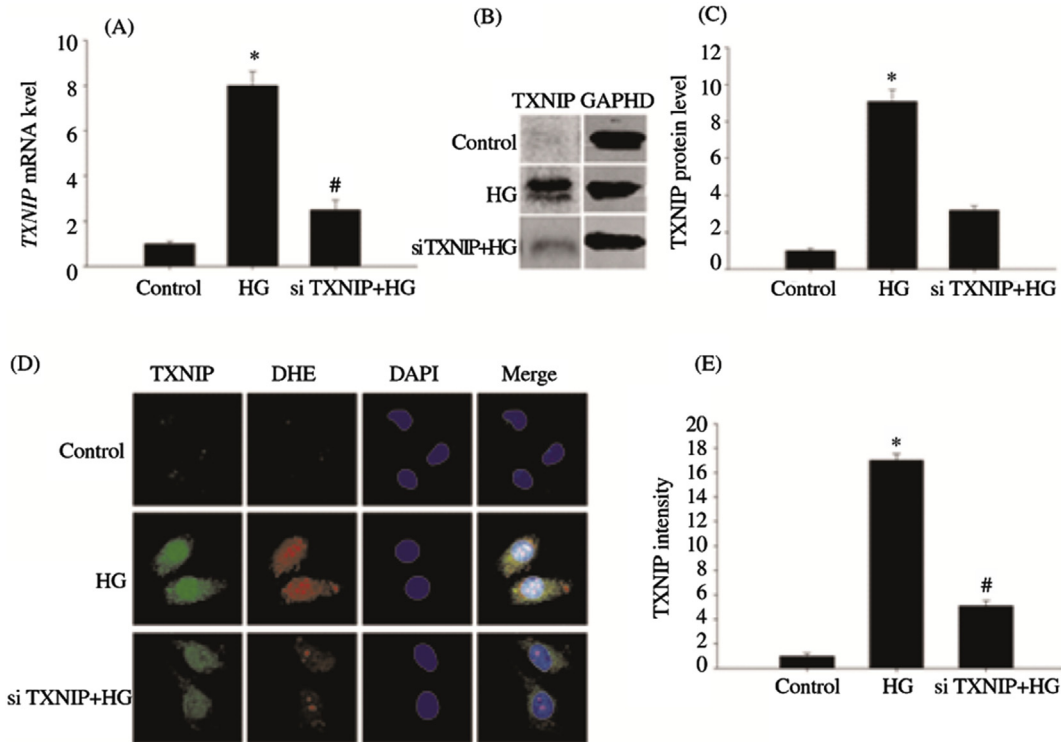
Figure 2. TXNIP expression levels in HUVEC in different groups.
A: *TXNIP* mRNA in HUVEC; B, C: TXNIP protein in HUVEC by Western blot; D, E: TXNIP protein in HUVEC by immunofluorescence method; [*]: Statistically significant difference between HG and control groups ($P < 0.05$); [#]: Statistically significant difference between siTXNIP and HG groups ($P < 0.05$).
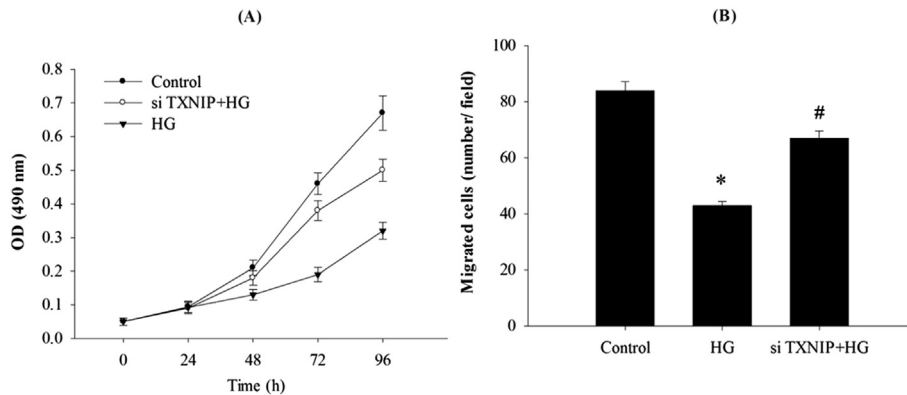

Figure 3. Cell proliferation and migration capability of HUVEC in different groups.
A: Cell proliferation changes; B: Cell migration capability; [*]: Statistically significant difference between HG and control groups ($P < 0.05$); [#]: Statistically significant difference between siTXNIP and HG groups ($P < 0.05$).
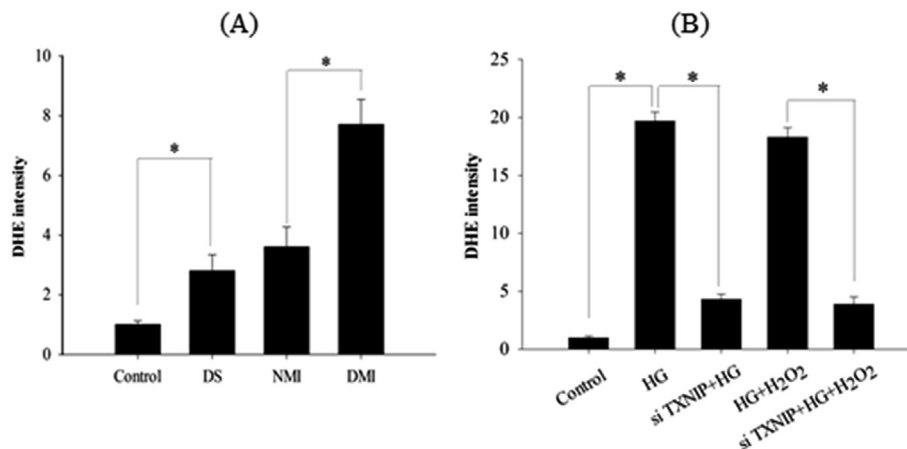

Figure 4. ROS levels in HUVEC and heart in different groups.
A: ROS levels in heart; B: ROS levels in HUVEC; [*]: Statistically significant difference between groups ($P < 0.05$).
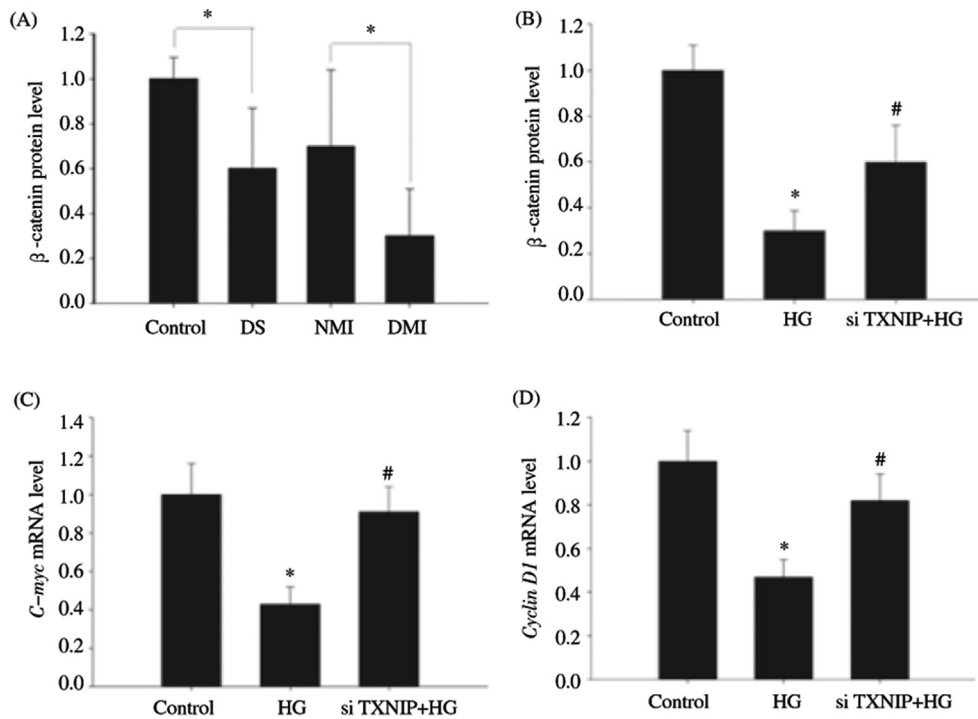
Figure 5. Expression levels of β-catenin in heart and β-catenin, *Cyclin D1* and *C-myc* in HUVEC in different groups.
A: β-catenin in heart; B, C, D: β-catenin, *Cyclin D1* and *C-myc* in HUVEC; [*]: Statistically significant difference between groups or between HG and control groups ($P < 0.05$); [#]: Statistically significant difference between siTXNIP and HG groups ($P < 0.05$).

4. Discussion

Diabetes is one of the major chronic diseases affecting people's health and there have been researches confirming that diabetes may affect the angiogenesis after occurrence of myocardial ischemia [15], and inhibit the formation of coronary collateral circulation [9,10], and these functions are closely related to endothelial cells. Therefore, researches on effect of endothelial cells on cardiovascular diseases are of great significance in exploring the pathogenesis of diabetes related diseases. The present study generated diabetic myocardial infarction models and HUVEC after high concentration glucose treatment to analyze and also illustrate the regulatory mechanism of TXNIP in diabetic myocardial infarction through Wnt/β-catenin signaling pathway.

As the key regulatory protein in diabetes, the mechanism of TXNIP has been the research focus in recent years. TXNIP belongs to the α inhibitory protein whose expression can be activated in high glucose environment and then the blood glucose can be regulated [16]. Anu *et al* reported the high expression of TXNIP in patients with diabetic nephropathy [17]; Chai *et al* reported the close connection between the regulatory function of melbine on glucose and its inhibition against the high expression of TXNIP [18]. The present study finds that compared with normal rats, diabetes would cause high expression of myocardial tissue and endothelial cells in heart and meanwhile the high glucose upregulates the expression level of TXNIP in HUVEC. All of the results show the positive correlation between TXNIP expression and incidence of diabetes. In contrast with rats with non-diabetic myocardial infarction, the TXNIP expression in myocardial infarction and heart endothelial cells in diabetes rats were significantly elevated, which may be related to the aggravated damage induced by myocardial ischemia under the state of

diabetes. It is further found in the present study that high glucose significantly decreased the proliferation and migration capability of HUVEC; and siTXNIP can reversely transcripted the inhibitory effect of high glucose on proliferation and migration capability of HUVEC, which confirms the above speculation. It is clear that the inhibitory effect of TXNIP on proliferation and migration capability under the state of diabetes may lead to impaired angiogenesis and further cause myocardial ischemia-induced damage.

Wnt/β-catenin signaling pathway is important in cell proliferation and others and researches show that this signaling pathway can be activated after occurrence of myocardial infarction [19,20], so as to repair the damaged myocardium [21]. Whether the inhibitory effect of TXNIP expression on cell proliferation is the result of regulatory function of TXNIP through Wnt/β-catenin signaling pathway? With an aim to solve the problem, the present study analyzed the hearts of rats with diabetic myocardial infarction and HUVEC after high glucose treatment, and found that the β-catenin expression levels in both heart and HUVEC were significantly lower than those in control group. In the meanwhile, the downstream protein of β-catenin, *Cyclin D1* and *C-myc* gene in HUVEC were significantly decreased and siTXNIP can reversely transcript the change. The study results show that TXNIP expression under the state of diabetes can down-regulate the activity of β-catenin so that the Wnt/β-catenin signaling pathway can play a regulatory role in cells.

ROS is the major production of mitochondrial respiration, whose level changes are important in cellular signal transduction, and occurrence as well as development of atherosclerosis, diabetes and so on [22–24]. Researches show that under the state of high glucose, TXNIP expression was significantly up-regulated so as to stimulate the cells to produce oxidative stress, to damage DNA *etc* [25], and inhibiting TXNIP

expression can slow down the cell apoptosis [26]. In the present study, diabetes up-regulated TXNIP expression and meanwhile increased ROS levels in rats and HUVEC, whereas siTXNIP can inhibit the production of ROS, which indicates that diabetes can aggravate the production of ROS after the occurrence of myocardial infarction and at the same time, that the regulatory function of TXNIP against β-catenin may be partially achieved through ROS level changes in cells.

In conclusion, the study results show that diabetic myocardial ischemia can increase the expression of TXNIP in myocardial infarction area and endothelial cells while the latter can regulate the β-catenin by changing ROS levels so as to affect the proliferation and migration of endothelial cells of myocardium, and so forth. As a matter of fact, TXNIP may act on Wnt/β-catenin signaling pathway through multiple ways including ROS, and other ways and the mechanisms of action still remain to be further explored.

Conflict of interest statement

We declare that we have no conflict of interest.

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