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The role of CTRP9 on Inhibition the High-glucose-induced Apoptosis of Myocardial Cells via Wnt/β-catenin Signal Pathway

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ABSTRACT

This study aimed to investigate the effect of CTRP9 regulating the Wnt/β-catenin signal pathway on the high-glucose-induced apoptosis of myocardial cells. For this purpose, high glucose was used to establish the myocardial cell apoptosis models on H9c2 cells which were later divided into 11 groups, with different treatments: NG group, NG+C group, NG+SKL group, NG+SKL+C group, NG+C59 group, HG group, HG+C group, HG+SKL group, HG+SKL+C group, HG+C59 group and HG+4h C group. Following the treatment, a TUNEL assay was applied to determine the apoptotic rate of cells, and RT-PCR and Western blot were carried out to determine the expression of targeted proteins or genes and the activity of the Wnt/β-catenin signal pathway. In comparison with the cells in the NG group, cells following the 48 hours of treatment with 25 mmol/L high glucose experienced an acute increase in the apoptotic rate, with upregulation of Caspase-3 and Bax and downregulation of Bcl-2. In addition, CTRP9 treatment for the high-glucose-treated myocardial cells partially reversed the effect of single treatment by high glucose, with manifestations of decreased apoptotic rate, downregulation of caspase-3 and Bax, upregulation of Bcl-2 and inhibition of Wnt/β-catenin signal pathway. Furthermore, SKL2001, the agonist of the Wnt/β-catenin signal pathway, was added into the high-glucose-treated cells and increased the apoptotic rate, with the activation of the Wnt/β-catenin signal pathway, which, however, was reversed by the treatment of CTRP9. In general, CTRP9, by inhibiting the activity of the Wnt/β-catenin signal pathway, can alleviate the high-glucose-induced apoptosis of myocardial cells.

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Introduction

Diabetes mellitus, a kind of metabolic disorder with increased blood glucose as the major feature, is induced by various pathogens, including genetic factors, immune and diet, *etc* (1). According to the latest report of the International Diabetes Federation (IDF), there have been 463 million adults with diabetes mellitus, almost 1 patient in every 11 people, and as estimated, this digit will surge to 578.4 million by the year 2030 (2). In the world, diabetes mellitus has become a popular disease with an increasing prevalence, severely threatening the health of human beings, wherein death caused by the complication of cardiovascular diseases takes up nearly 70% to 80% of the death rolls of diabetes mellitus (3).

Diabetic cardiomyopathy (DCM), as a severe, independent heart complication, is the major cause of heart failure and death of patients (1). The pathogenesis of DCM, as evidenced by the current work, is quite complicated, and it is speculated that DM-induced metabolic disorder induces the increases in blood glucose and triglyceride, further activating the local renin-angiotensin system, oxidative stress and inflammatory responses, which gives rise to the damage and apoptosis of myocardial cells and eventually alters the myocardial structure and function (2). However, the pathogenesis of DCM has yet been to be elucidated, so the in-depth research on the pathogenesis of high-glucose-induced apoptosis of myocardial cells is of great significance for prophylaxis and treatment of DCM (1).

Wnt/β-catenin signal pathway, a classic, highly conservative signal pathway, is reported to be involved in the growth and differentiation of myocardial cells, thereby playing a key role in the myocardial damage (3). Wnt2, as reported, is upregulated after myocardial infarction (4), while the accumulation of β-catenin in cytoplasm promotes the

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atrophic progression of myocardial cells (5), and the administration of an inhibitor of Wnt/β-catenin signal pathway can improve the ventricular remodeling significantly (6, 7). It is also reported that the Wnt/β-catenin signal pathway is associated with diabetes mellitus, and in the diabetes mellitus rats with myocardial fibrosis present with the upregulation of Wnt7b (8). In addition, the overexpression of connective tissue growth factor in the diabetic retinopathy of diabetes mellitus rats is reversed by the inhibition of the Wnt/β-catenin signal pathway, and the anomaly in the Wnt/β-catenin signal pathway can also give rise to the disorder in glucose metabolism and insulin resistance (9). Thus, we infer that the Wnt/β-catenin signal pathway is associated with diabetes mellitus and myocardial damage.

C1q/TNF-related protein 9 (CTRP9) is a newly found adipocytokine. Mouse genome harbors the *CTRP9* (10), while in human beings, *CTRP9* is categorized into two subtypes, *CTRP9A* and *CTRP9B*, and proteins encoded by the two subtypes are highly consistent in the amino acid sequences. CTRP9, a homologous protein of adiponectin, shares the molecular structure in high degree with the adiponectin, with the critical energy-regulation function (11, 12). The expression of CTRP9 in the heart, exceeding that of adiponectin, is negatively regulated by various factors, including type 2 diabetes mellitus and obesity, and is believed to be a key mediator underlying diabetes mellitus and cardiovascular disorder. Existing evidence has shown that CTRP9 can ameliorate ventricular remodeling after myocardial infarction (13). Therefore, we infer that CTRP9 may play a role in the high-glucose-induced myocardial cell apoptosis by inhibiting the activity of Wnt/β-catenin signal pathway.

Hence, in this study, we aimed to clarify the effect of CTRP9 by regulating the activity of the Wnt/β-catenin signal pathway on the high-glucose-induced apoptosis of myocardial cells, so as to provide theoretical evidence for the clinical prophylaxis and treatment of DCM.

Materials and methods

Culture of myocardial cells

In this study, the H9c2 cell line, a rat myocardial cell line provided by the Cell Bank (Shanghai) of the Chinese Academy of Sciences, was sustained at DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 and passaged in 0.25% trypsin. Following being starved in serum-free DMEM for 24 h, cells were divided into normal-glucose (NG) groups and high-glucose (HG) groups for the following experiments. For normal groups, cells were further divided into the Control group (NG group; cells were cultured at medium supplemented with 5.5 mM glucose for 24 h), NG + CTFP9 group (NG + C group; cells were cultured at medium supplemented with 5.5 mM glucose and 5μg/ml CTRP9 for 24 h), NG + SKL2001 group (NG + SKL group; cells were cultured at medium supplemented with 5.5 mM glucose and 5 μg/mL SKL2001 for 24 h), NG + SKL2001 + CTRP9 group (NG + SKL + C group; cells were cultured at medium supplemented with 5.5 mM glucose and 5μg/ml SKL2001 and CTRP9 for 24 h). For HG groups, cells were divided into the Control group (HG group; cells were cultured at medium supplemented with 25 mM glucose for 24 h), HG + CTFP9 group (HG + C group; cells were cultured at medium supplemented with 25 mM glucose and 5μg/ml CTRP9 for 24 h), HG + SKL2001 group (HG + SKL group; cells were cultured at medium supplemented with 25 mM glucose and 5μg/ml SKL2001 for 24 h), HG + SKL2001 + CTRP9 group (HG + SKL + C group; cells were cultured at medium supplemented with 25 mM glucose and 5 μg/mL SKL2001 and CTRP9 for 24 h), HG + Wnt-C59 group (HG + C59 group; cells were cultured at medium supplemented with 25 mM glucose and 5μg/ml Wnt-C59 for 24 h) and HG + 4 h CTRP9 group (HG + 4h C group; cells were firstly incubated with 5μg/ml CTRP9 for 4 h and then cultured in medium supplemented with 25 mM glucose for 24 h).

TUNEL staining

Cells on the slides were rinsed in PBS twice, 3 min/wash, and then incubated with proteinase K for 20 min at 37°C, which was terminated by rinsing cells in PBS twice, 5 min/wash. TUNEL mixture

was then prepared by mixing the 50μl TdT with 450μl fluorescein-labeled dUTP. Thereafter, on the dry slides, 50μl TUNEL mixtures were added into the cells, while for the negative control, cells were treated only with 50μl fluorescein-labeled dUTP, followed by incubation of cells under the coverslip or plastic membrane for 1 h at 37°C in a dark, wet box. Following 3 washes in PBS, DAPI staining solution was added on the cells, followed by incubation of cells under the coverslip or plastic membrane for 1 h at 37°C in a dark, wet box. Again, slides were placed in PBS for being rinsed three times, and cells were mounted by using the anti-cancellation mounting agent. Under the fluorescent microscope, apoptotic cells were observed and photographed, and the changes in apoptosis were evaluated as per the morphological features of cells.

Real-time PCR (RT-PCR)

TRIzol method was applied to extract the total RNA. In brief, cells in different groups were collected into the Eppendorf (EP) tubes containing 1 mL TRIzol and placed for 5 to 10 min; then, cells were mixed with 0.2 mL chloroform sufficiently and placed on ice for 3 min, followed by centrifuge at 12,000 rpm for 15 min at 4°C, where the upper aqueous layer was collected into a new EP tube to be mixed with 0.5 mL isopropanol on ice for 10 min; thereafter, the mixture was centrifuged at 12,000 rpm and 4°C for 15 min, and with the supernatant being discarded, sediment was mixed with 1 mL 75% icy ethanol, followed by centrifuge at 12,000 rpm and 4°C for 5 min; sediment after the centrifuge was dried for a while in the air and dissolved in DEPC water, where 2μl RNA was taken to measure the concentration and purity of total RNA using the ultraviolet spectrometer.

Reverse transcription was carried out according to the instruction of TIANScript RT KIT. In brief, the first part of a reaction system (50μl) was prepared and heated at 70°C for 5 min and cooled on ice for 2 min, followed by the instant centrifuge to collect the supernatant, where reagent in the second part was added and mixed sufficiently, followed by water bath at 25°C for 10 min, 42°C for 10 min and 95°C for 5 min; then, the system was diluted to 50μl by adding RNase-free ddH_2O for the following experiment or being stored at -20°C.

The reaction system was constructed according to the instruction of SuperReal PreMix Plus (SYBR Green) and carried out on the qRT-PCR apparatus in the following condition: Pre-denaturation at 95°C for 15 min and 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 30 s and extension at 72°C for 30 s. Relative quantification analysis was carried out using the method of $2^{-\triangle\triangle CT}$.

Western blot

Proteins, after being extracted from samples, were subjected to the measurement of concentration and mixed with 4× loading buffer to be heated at 95°C for 10 min. Proteins were separated in the electrophoresis in SDS-PAGE and then transferred to the PVDF membrane in following transferring time: C-myc, 50 min; Wnt-3a, 33 min; Bax, 20 min; Bcl-2, 23 min; β-catenin, 66 min; Caspase-3, 23 min. The unoccupied sites on the PVDF membrane were then blocked in non-fat milk at room temperature for 2 h. Primary antibodies (dilution for C-myc, 1:500; Wnt-3a, 1:600; Bax, 1:600; Bcl-2, 1:300; β-catenin, 1:300; Caspase-3, 1:400) were added on the membrane for incubation at 4°C overnight, followed by three washes in 1× PBST, 10 min/wash. Corresponding secondary antibodies at a dilution of 1:3000 were added to the membrane for incubation at room temperature for 90 min, followed by three washes in 1×PBST, 10 min/wash. Then, the membrane was placed in the ECL reagent to develop the bands of proteins, and with the intensity of the β-actin band as an endogenous reference, Tanon Gis software was used to analyze the intensity of bands of targeted proteins.

Statistical methods

SPSS 21.0 software was used to perform the data analysis. Measurement data in normal distribution were expressed by mean ± standard deviation (mean ± SD), and the differences among groups were validated by the One-Way ANOVA. $P < 0.05$ suggested that the difference had statistical significance.

Results and discussion

High glucose induces the apoptosis of myocardial cells

Results of TUNEL staining (Figure 1) showed that in the HG group, TUNEL-positive cells, presenting with the green fluorescent signals, were significantly more than those in the NG group, and the further analysis revealed that in the HG group, the apoptotic rate of myocardial cells was 0.50%, significantly higher than 0.12% in the NG group, suggesting that high glucose treatment increased the apoptotic rate of myocardial cells (P<0.05).

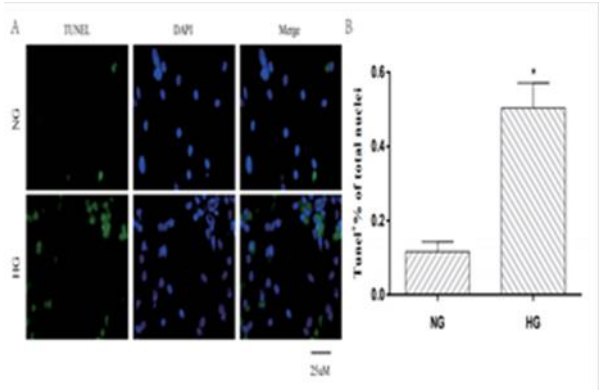

Figure 1. TUNEL staining results of myocardial cells in NG group and HG group; A, Results of TUNEL staining to reflect the apoptosis of myocardial cells in the NG group and HG group; B, Comparison of the apoptotic rates of myocardial cells in the NG group and HG group; * P<0.05 *vs.* the NG group.

Results of RT-PCR (Figure 2) indicated that in the HG group, Caspase-3 and Bax were significantly upregulated, significantly higher than those in the NG group (all P<0.05), while the Bcl-2 was lower than that in the NG group (P<0.05), suggesting that high glucose treatment resulted in the apoptosis of myocardial cells.

CTRP9 inhibits the high glucose-induced apoptosis of myocardial cells

Results of TUNEL staining (Figure 3) showed that the quantity of TUNEL-positive cells in the HG + C group was far more than that in the HG group, while the further quantitative analysis revealed that in the HG group, the apoptotic rate of myocardial cells was 0.50%, significantly higher than 0.30% in the HG + C group (all P<0.05), indicating that under the high-glucose treatment, CTRP9 reduced the apoptotic rate of myocardial cells.

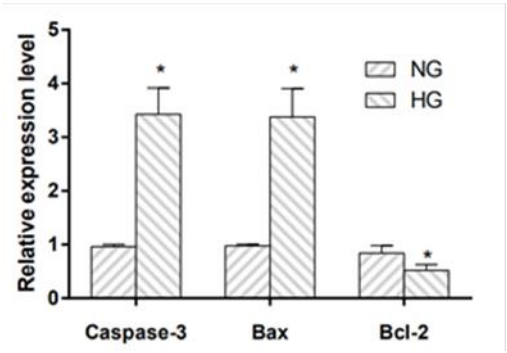

Figure 2. RT-PCR results of the expression of apoptotic proteins in the NG group and HG group: RT-PCR results to reflect the differences in the expression of Caspase-3, Bax and Bcl-2 between the NG group and HG group; * P<0.05 *vs* the NG group

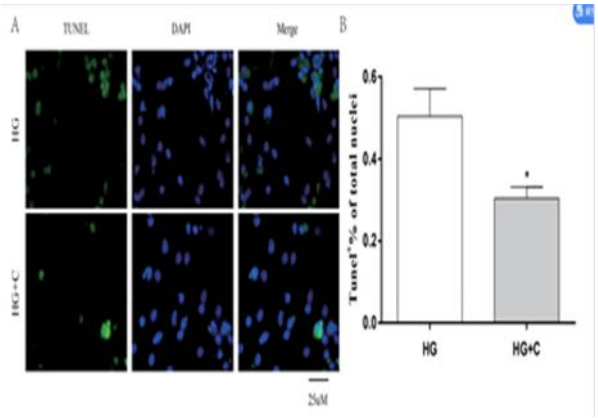

Figure 3. TUNEL staining results of the apoptosis of myocardial cells in the HG group and HG + C group; A, TUNEL staining results to reflect the apoptosis of myocardial cells in the HG group and HG + C group; B, Comparison of the apoptotic rate of myocardial cells between the HG group and HG + C group; * P<0.05 *vs.* the HG group

Results of RT-PCR (Figure 4) indicated that in comparison with the cells of the NG group, cells in the NG + C group presented with no significant differences in the expression of Caspase-3; while the expression of Caspase-3 in the HG + C group and in the HG + 4h C group was significantly lower than that in the HG group (all P<0.05), and no significant difference was identified in comparison between the HG + C group and the HG + 4h C group (Figure 4A). Bax showed expression profiles similar to those of Caspase-3 (Figure 4B). However, in comparison with the NG group, Bcl-2 in the NG + C group was downregulated remarkably (P<0.05), but when it came

to the treatment of high glucose, no significant difference was shown in the comparison of Bcl-2 expression between the HG group and HG + C group, while in the HG + 4h C group, Bcl-2 was upregulated evidently, higher than that in the HG group (P<0.05) (Figure 4C). Thus, CTRP9 could inhibit the high glucose-induced apoptosis of myocardial cells.

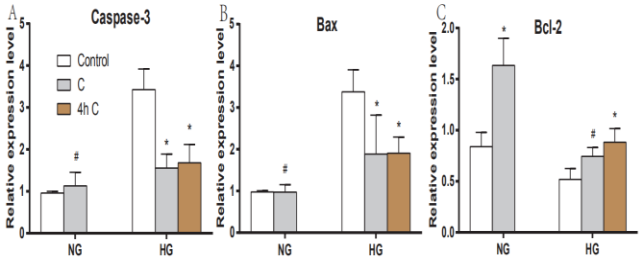

Figure 4. RT-PCR results of the apoptotic proteins in each group. RT-PCR results to reflect the differences in the expression of Caspase-3, Bax and Bcl-2 between the HG group, HG + C group and HG + 4h C group; * *P*<0.05 *vs.* the NG or HG group; # *P*>0.05 *vs.* the NG or HG group.

CTRP9 curbs the high glucose-induced apoptosis of myocardial cells by Wnt/β-catenin signal pathway

Results of RT-PCR (Figure 5) indicated that in comparison with the NG group, Wnt-3a in the NG + C group demonstrated no significant variation, while the expression of Wnt-3a in the HG + C group and in the HG + 4h C group was significantly lower than that in the HG group (all *P*<0.05), but no significant difference was found between the HG + C group and HG + 4h C group (Figure 5A). Expression of c-Myc was similar to that of Wnt-3a (Figure 5C). In comparison with the NG group, β-catenin in the NG + C group was downregulated sharply (P<0.05), and when it came to the high-glucose environment, in comparison with the NG group, β-catenin was also downregulated in the HG + C group and in the HG + 4h C group (all P<0.05) (Figure 5B).

Thus, in the high-glucose environment, CTRP9 inhibits the expression of key proteins of Wnt/β-catenin signal pathway. Similar results were also demonstrated in the experiments of Western blot (Figure 6).

Results of TUNEL staining (Figure 7) also showed that the proportion of TUNEL-positive cells in the HG + SKL group was much higher than that in the HG group, and as evidenced by the further quantitative analysis, the apoptotic rate of myocardial cells was 0.50%, significantly lower than 0.70% in the HG + SKL group (P<0.05), indicating that SKL increased the apoptotic rate of myocardial cells in the high-glucose environment.

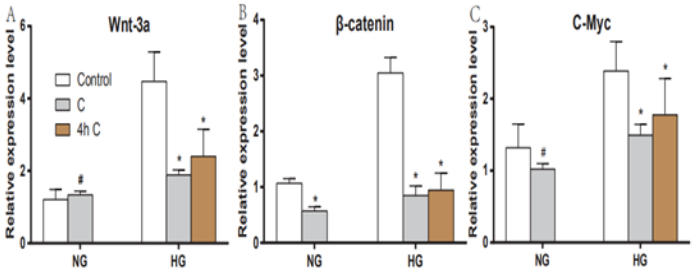

Figure 5. RT-PCR results of mRNA expression of Wnt/β-catenin signal pathway in each group; RT-PCR results to reflect the differences in the mRNA expression of Wnt-3a (A), β-catenin (B) and c-Myc (C) in Wnt/β-catenin signal pathway between NG group, NG+C group, HG group, HG + C group and HG + 4h C group; * *P*<0.05 *vs.* the NG or HG group; # *P*>0.05 *vs.* the NG or HG group.

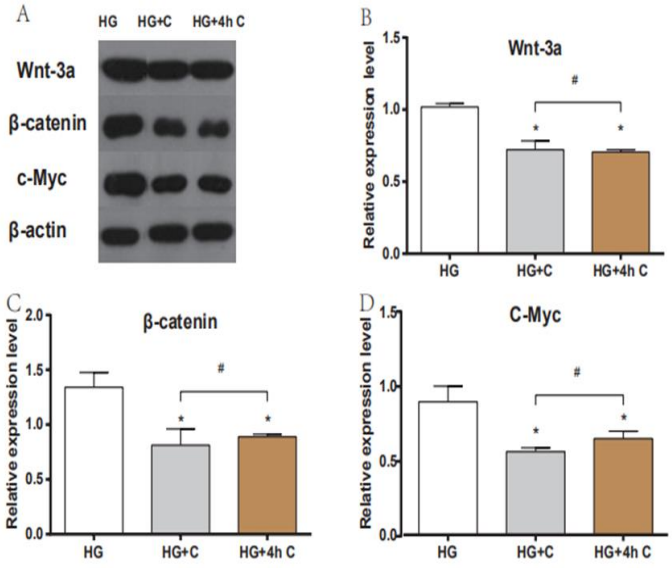

Figure 6. Western Blot results of protein expression in the Wnt/β-catenin signal pathway; RT-PCR results to reflect the differences in the expression of Wnt-3a (A), β-catenin (B) and c-Myc (C) in Wnt/β-catenin signal pathway between the NG group, NG + C group, HG group, HG + C group and HG + 4h C group; * P<0.05 vs. the NG or HG group; # P>0.05 vs. the NG or HG group.

Besides, the proportion of TUNEL-positive cells in the HG + SKL + C group was much lower than that in the HG + SKL group, and according to the quantitative analysis, the apoptotic rate of myocardial cells in the HG + SKL + C group was 0.53%,

significantly different from that of the HG + SKL group (P<0.05). Hence, in the high-glucose environment, CTRP9 could reverse the SKL-induced increase of apoptotic rate of myocardial cells.

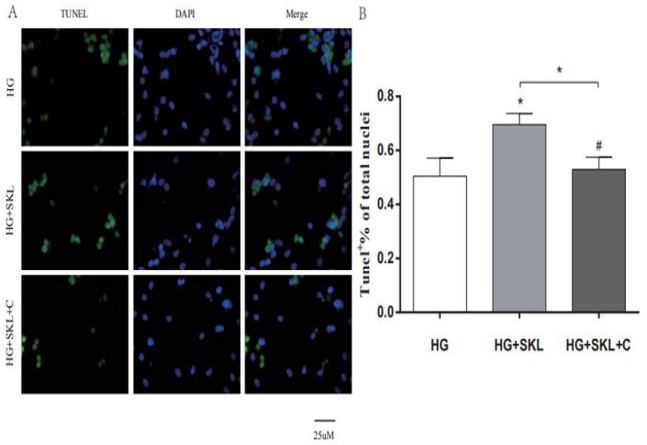

Figure 7. TUNEL staining results of apoptosis of myocardial cells in each group; A, TUNEL staining results to observe the apoptosis of myocardial cells in the HG group, HG + SKL group and HG + SKL + C group; B, Comparison of the apoptotic rates of myocardial cells between the HG group, HG + SKL group and HG + SKL + C group; * *P*<0.05 *vs.* the NG or HG group; # *P*>0.05 *vs.* the NG or HG group.

Results of RT-PCR (Figure 8) indicated that in comparison with the NG group, Wnt-3a in the HG + SKL group was upregulated evidently, but in the HG + C group was downregulated (all P<0.05); in comparison with the HG + SKL group, Wnt-3a in the HG + SKL + C group was downregulated evidently (P<0.05). Wnt-3a manifested the expression profiles in the high glucose environment similar to those in the normal glucose environment (Figure 8A). The expression of β-catenin (Figure 8B) and c-Myc (Figure 8B) was similar to that of Wnt-3a.

DCM, as a particularly severe cardiac complication of diabetes mellitus, is a kind of secondary myocardial damage (14). From the long-term perspective, patients with DCM usually suffer from the insufficient use of glucose, increased steatolysis of fat tissue and accumulation of free fatty acid, which, more importantly, give rise to the oxidative stress and mitochondrial DNA damage that contribute to the damage to the endothelial cells of myocardial capillary, or even apoptosis of cells; since myocardial cells possess no regeneration ability, patients may face the heart failure eventually (15).

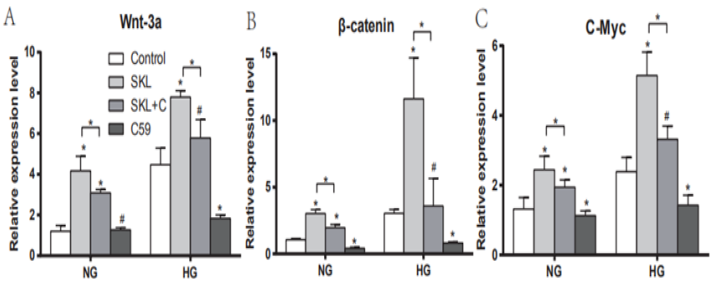

Figure 8. RT-PCR results of the mRNA expression of Wnt/β-catenin signal pathway in each group; RT-PCR results to reflect the differences in the mRNA expression of Wnt-3a (A), β-catenin (B) and c-Myc (C) in the Wnt/β-catenin signal pathway between the NG group, NG+SKL group, NG+SKL+C group, NG+C59 group, HG group, HG+SKL group, HG + SKL + C group and HG+C59 group; * *P*<0.05 *vs.* the NG or HG group; # *P*>0.05 *vs.* the NG or HG group.

Existing data have shown that high glucose can induce the apoptosis of myocardial cells. Ernest Adeghate *et al*. constructed the DCM models on rats and found that treatment of high glucose increased the apoptotic rate of myocardial cells (16). Tsai *et al*. utilized cultured myocardial cells and found that after stimulation of high glucose, the apoptotic rate of myocardial cells was increased evidently (17). Likewise, we, in this study, found that the apoptotic rate of myocardial cells was increased after the high glucose treatment. Since Caspase-3 is the key protein in apoptosis, and Bax and Bcl-2 are the major pro-apoptotic and anti-apoptotic proteins, we further detected the expression of Caspase-3, Bax and Bcl-2, and as a result, Caspase-3 and Bax had upregulation in the mRNA expression, while Bcl-2 manifested the opposite change, which, taken together, showed that high glucose treatment increased the apoptosis of myocardial cells.

Studies in recent years have shown that the CTRPs family, consisting of 15 members with a highly conserved structure, may represent the key mediator between diabetes mellitus and cardiovascular diseases (18). According to the current evidence, CTRP1 is mainly distributed in muscle, fat tissues and the heart, CTRP2, CTRP3 and CTRP12 mainly in the fat tissues, CTRP13 in fat tissues and brain, while CTRP9 in the matrix cells of vessels and those of fat tissues. As a superfamily of adipocytokines, CTRPs are extensively involved in vasodilation, glucose

metabolism and inflammatory responses. For instance, CTRP3 inhibits the myocardial fibrosis, and its content is correlated with the stenosis of the coronary artery, while in the diabetes mellitus rats, CTRP3 is also downregulated significantly in the heart tissue; CTRP12 could facilitate the secretion of insulin to get involved in the glucose metabolism and also inhibit the inflammatory responses of fat tissues. CTRP9, as the homologous protein sharing the structure of adiponectin, also shares the function of adiponectin in metabolism regulation (19). In this study, myocardial cells in the high glucose environment were treated by CTRP9, and CTRP9 was found to be able to inhibit the apoptosis of myocardial cells in the high glucose environment, with the manifestation of a decrease in the apoptotic rate of myocardial cells, downregulation of mRNA expression of Caspase-3 and Bax and upregulation of mRNA of Bcl-2. Ma *et al*. (18) in the obese models of mice found that overexpression of CTRP9 could reduce the blood glucose and insulin by increasing the use of glucose. Similarly, in H9c2 cells, overexpression of CTRP9 reduced the area of myocardial infarction (20). Also, CTRP9, as reported, can suppress the high glucose-induced atrophy of myocardial cells. Hence, we aimed to clarify the mechanism regulating the inhibitory effect of CTRP9 on the apoptosis of myocardial cells in the high glucose environment.

Further detections were carried out to determine the expression of relative molecules in the Wnt/β-catenin signal pathway, and the findings revealed that in the high glucose environment, CTRP9-treated myocardial cells presented with the downregulation of mRNA and protein expression of Wnt-3a, β-catenin and c-Myc, suggesting that CTRP9 inhibited the expression of Wnt/β-catenin signal pathway. However, administration of SKL2001, the agonist of the Wnt/β-catenin signal pathway, increased the apoptotic rate of myocardial cells, with upregulation of mRNA and protein expression of Caspase-3 and Bax and downregulation of Bcl-2. Co-treatment of SKL2001 and CTRP9 resulted in an acute decrease in the apoptotic rate of myocardial cells, confirming that CTRP9 is able to reduce the high-glucose-induced apoptosis of myocardial cells by inhibiting the Wnt/β-catenin signal pathway. The Wnt/β-catenin signal pathway has been focused extensively on myocardial damage for its role in the regulation of the transcription of target genes (21). Zelaraya *et al*. also noted that the inhibition of β-catenin is conducive to the left ventricular remodeling (6), while the anomaly in the Wnt/β-catenin signal pathway gives rise to the metabolic syndrome and insulin resistance (22-24). The findings above are closely associated with the pathogenesis of DCM (15). Our work also demonstrated that in the high glucose environment, CTRP9 can inhibit apoptosis via the Wnt/β-catenin signal pathway.

In conclusion, CTRP9, by inhibiting the activity of the Wnt/β-catenin signal pathway, can alleviate the high-glucose-induced apoptosis of myocardial cells, which is expected to provide new theoretical evidence for treatment of DCM.

Acknowledgments

None

Conflict interest

The authors declare no conflict of interest.

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