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A study of the effects of SGLT-2 inhibitors on diabetic cardiomyopathy through miR-30d/KLF9/VEGFA pathway

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Abstract. – OBJECTIVE: The aim of this study was to observe the protective effects of SGLT-2 inhibitors on diabetic cardiomyopathy.

MATERIALS AND METHODS: After constructing a diabetic rat model, the effects of SGLT-2 (Sodium-Glucose Cotransporter-2) inhibitors and miR-30d on cardiac function in rats were investigated by cardiac echocardiography, hematoxylin-eosin (HE) staining, and immunohistochemical methods. At the same time, changes in autophagy levels in rats were detected by Western blot (WB) experiments.

RESULTS: SGLT-2 inhibitors improved the cardiac function of diabetic rats, and alleviated the pathological damage of myocardial tissue. Besides, knocking down miR-30d prevented the decrease of cardiac function in diabetic rats. Moreover, miR-30d could regulate the expression of the KLF9/VEGFA pathway and inhibit autophagy in rats.

CONCLUSIONS: SGLT-2 inhibitors can regulate the autophagy level in diabetic rats through the miR-30d/KLF9/VEGFA pathway, thereby improving cardiac function.

Key Words:
SGLT-2 inhibitor, MiR-30d, Diabetic cardiomyopathy, Autophagy.

Introduction

Diabetes mellitus (DM) is a common chronic disease worldwide and one of the major risk factors for cardiovascular disease (CVD)[1]. It is estimated that there will be 300 million people with diabetes worldwide by 2025, with a prevalence of about 5.4%. However, even if the coronary arterial state and blood pressure are normal, diabetes in a small number of patients will develop into heart failure (HF), which is clinically known as diabetic cardiomyopathy (DCM)[2]. Currently, DCM refers to heart disease caused by DM-induced changes in myocardial primary metabolic disorders and microvascular disease, excluding cardiac valve disease, congenital heart disease, hypertension, alcoholism, and coronary heart disease. Although several mechanisms have been postulated, their pathogenesis remains controversial. In addition, DCM usually appears for a long subclinical period before diagnosis[3,4]. Generally speaking, DCM includes three phases asymptomatic (early) phase, diastolic dysfunction phase and systolic dysfunction (late phase) phase. There is a close relationship between the decrease in autophagy activity and DCM, which is one of the main causes of DCM. Autophagy is an important intracellular degradation mechanism that is highly conserved in eukaryotic cells and maintains the stability of the intracellular environment. However, the mechanism of autophagy regulation of cardiomyocytes in high glucose environment is not fully understood.

Intercellular electrical coupling in the heart occurs primarily through gap junctions. These membrane structures consist of intercellular hemichannels formed by connexin components that connect adjacent cells and allow electrochemical communication. Connexin 43 is expressed in the ventricles[5]. Changes in the expression of connexin 43 are associated with various pathological conditions, such as myocardial ischemia, HF, hypertrophy, and arrhythmias[6-8] and may impair the gap junctional communication of diabetic heart cells, providing a basis for arrhythmias in a variety of HFs[9]. In addition, diabetic patients are prone to ventricular arrhythmias due to con-

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duction disorders such as delayed conduction or block. Therefore, the worsening of diabetic heart disease may be caused by the impairment of gap junction function, because gap junction plays a major role in intercellular pulse propagation. A decrease in the expression of connexin 43 in the heart of diabetic patients leads to a decrease in electrochemical communication, and the autophagy process is involved in the regulation of connexin 43 expression in the heart[10].

MicroRNAs (miRNAs), small endogenous non-coding RNA molecules, play a regulatory role through targeted mRNA cutting or translation inhibition. As miRNAs mainly target mRNA, they may affect the output of many protein-coding genes[11]. MiRNAs are involved in the pathophysiology of DCM[12], and miRNA-133 and miRNA-1 are abundantly expressed in normal cardiomyocytes. Of note, inhibition of miRNA-133 has been found to cause cardiac hypertrophy[13] and miRNA-373 is downregulated in streptozotocin (STZ)-induced models, leading to cardiac hypertrophy[14]. MiRNA-34a and other miRNAs are up-regulated in diabetic heart[15-17], and miR-30c is involved in DCM by regulating Beclin1 protein expression[18]. MiR-30d is a member of the miR-30 group, located on human chromosome 8, which plays an important regulatory role in the process of tumor formation[19], mitochondrial division[20] and cell apoptosis[21]. However, there are few studies on the role of miR-30d in DCM. This gives us a hint, how is miR-30d expressed in DCM cardiomyocytes? If the expression is abnormal, does it participate in the pathogenesis of DCM?

Currently, there are no clear studies focusing on the role of sodium-glucose cotransporter-2 (SGLT-2) inhibitor in T2DM patients with diastolic dysfunction. Considering the increasing threat of HF to T2DM patients, more specific studies should be conducted to better understand the effects of SGLT-2 inhibitors on diastolic and systolic dysfunction. Two large investigations have shown that the SGLT-2 inhibitors, Englitazone and Kanglegine, reduce cardiovascular mortality in patients with type 2 diabetes[22,23]. Englitazone has been shown to have anti-inflammatory and antioxidant effects in experimental animal models and reduced Na^+ and Ca^{2+} levels in isolated ventricular myocytes. Of note, Englitazone is able to inhibit the Na^+/H^+ exchanger (NHE) and activate the Na^+/Ca^{2+} exchanger[24].

In conclusion, SGLT-2 inhibitors have positive effects on lowering blood glucose in a safe and effective manner without affecting body weight. However, whether it has a preventive effect on DCM has not yet been fully clarified, and the specific mechanism of its effect on DCM remains to be further explored.

Materials and Methods

Experimental Animals

8-week-old male clean Sprague Dawley (SD) rats (Huafukang, Beijing, China) weighing 250±10 g were kept in the Specific Pathogen Free (SPF) animal room of the Chinese Academy of Medical Sciences. The ambient temperature was set at 20-24°C, the light and dark cycle was 12 h, and the ventilation was good. SD rats were randomly divided into cages, 5 per cage. The litter was changed every 2 days, and the rats ate and drunk freely. This investigation was approved by the Animal Ethics Committee of Inner Mongolia Medical University Animal Center.

Modeling Diabetic Rats

SD rats were adaptively fed for one week. The NC group was fed with a normal diet, while the model group was fed with a high-fat diet (Boaigang, Beijing, China). After 10 weeks of fasting, the OGTT test was performed for 12 h. The results of the experiment were insulin resistance, and the modeling could continue. The rats in the model group were fasted for 12 h, weighed, and injected with STZ solution (concentration: 30 mg/kg, Boaigang, Beijing, China) in the tail vein. The injections were given every other day for a total of 5 injections, without eating or drinking. In the NC group, sodium citrate solution was injected in the same way. On the third day after the injection, a random tail vein blood glucose measurement was started, and the blood glucose level was 2 times or more) ≥16.7 mmol/L or one fasting blood glucose ≥8.0 mmol/L, which could be considered as successful modeling of diabetes.

Echocardiographic Evaluation of Rat Heart Structure and Function

After anesthetizing the rats with isoflurane, the fur on the left anterior thorax was shaved, the coupling agent was applied, and small animals were tested for high-resolution ultrasound with cardiac function in each group. The frequency of the probe was 15 Hz, and the left ventricular long-axis section near the sternum and the left ventricular short-axis section at the level of the papillary muscle were measured. M-mode echo-

cardiography (Nuohai Life Science, Shanghai, China) was used to measure the Left ventricular wall end-diastolic thickness (LVIDd), Left ventricular wall end-shrinkage thickness (LVIDs), ejection fraction (EF), and shortened fraction (FS). Continuous wave Doppler echocardiography (Nuohai Life Science, Shanghai, China) was applied to determine the maximum E-wave velocity, A-wave velocity, and E/A ratio of the mitral valve orifice.

Specimen Collection

After fasting for 12 h, the blood was collected by eyeball extraction. Subsequently, the rats were sacrificed by cervical descaling, and then the serum was centrifuged at 4°C, 1000 rpm, and stored at -80°C in a low-temperature refrigerator (HaoXin, Quanzhou, China). After all rats were sacrificed, the chest cavity was opened and the heart was removed. The tissue around the heart was removed, and the residual blood was rinsed with saline and then blotted with filter paper. After the whole weighing, the apical part was cut and fixed in 4% paraformaldehyde (Bio-Rad, Hercules, CA, USA) fixative solution, and the subsequent paraffin embedding was performed. Finally, the remaining heart tissues were stored in sterile cryopreservation tubes and immediately stored in liquid nitrogen.

Determination of Blood Biochemical Parameters In Rats

Automatic biochemical analyzer (Roche, Basel, Switzerland) was used to determine aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL-C), low density lipoprotein (LDL-C), blood urea nitrogen (BUN), and serum creatinine (Scr) levels.

Hematoxylin-Eosin (HE) Staining

The tissues were removed from the 4% paraformaldehyde fixative, dehydrated, embedded in paraffin, and then cut into 4 μm sections. Thereafter, the sections were dewaxed through a gradient of xylene (Roche, Basel, Switzerland) and ethanol (Roche, Basel, Switzerland) and immersed in the hematoxylin stain (JianCheng, Nanjing, China), followed by 1% ethanol hydrochloride. Next, the slides were dipped in 0.5% eosin staining solution and then sequentially dehydrated in gradient ethanol and xylene. Finally, the film was sealed with neutral gum and dried under the microscope (Olympus, Tokyo, Japan).

Immunohistochemical Staining

Several 4 μm slices were taken and boiled in citrate buffer (Bio-Rad, Hercules, CA, USA). After natural cooling, 3% H_2O_2 (Hengjian, Guangzhou, China) was added for incubation to inactivate endogenous peroxidase activity. Then, connexin43 antibody (1:500, Abcam, Cambridge, MA, USA) was added for incubation overnight at 4°C. The next day, the slides were incubated with secondary antibodies (1:1000, Abcam, Cambridge, MA, USA) for 1 h. Next, diaminobenzidine (DAB) color development solution (Jincheng, Nanjing, China) was added and the degree of color development was observed under the microscope (Olympus, Tokyo, Japan). The nuclei were then stained with hematoxylin dye and differentiated with 0.5% alcohol hydrochloride. After that, the slides were placed in gradient ethanol and xylene for dehydration. Finally, the slides were sealed with neutral gum (Hengjian, Guangzhou, China), dried and observed under the microscope.

Real-Time Quantitative Polymerase Chain Reaction

A proper amount of rat myocardial tissue was put in an enzyme-free EP 1.5 mL Eppendorf (EP) tube (Hamburg, Germany), and the total RNA was extracted using the TRIzol method (Thermo Fisher Scientific, Waltham, MA, USA) 1 μg of which was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) under the following conditions: 50°C 45 min and 85°C 5 min. After the reverse transcription was completed, the obtained cDNA was used for the next PCR reaction. U6 was used as the internal reference for detection of miR-30d. PCR and data analysis were performed in the vii A7 Quantitative PCR System (Applied Biosystems, Foster City, CA, USA). The $2^{-\Delta\Delta CT}$ method was applied to calculate miR-30d and the relative expression level of microRNAs. Primers used were shown in Table I.

Western Blot (WB)

Radioimmunoprecipitation assay (RIPA) protein lysate (Camilo Biological, Nanjing, China) was used to extract myocardial tissue protein, the supernatant was centrifuged, and the protein was quantified by bicinchoninic acid (BCA) method (Jincheng, Nanjing, China). Then, 5 ×sodium dodecyl sulphate (SDS) loading buffer (Camilo Biological, Nanjing, China) was added, and the protein was denatured by heating at 100°C for 5 min, followed by polyacrylamide coagulation

Table I. Real time PCR primers.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
MiR-30d	GTCGTATCCAGTGCAGGGTC	GAGAGGAGAGGAAGAGGGAA
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

gel electrophoresis. After the electrophoresis, protein was transferred using a polyvinylidene difluoride (PVDF, Thermo Fisher Scientific, Waltham, MA, USA) membrane, sealed with 5% skimmed milk powder and incubated with the corresponding antibodies (LC3-II, Abcam, Cambridge, MA, USA, Rabbit, 1:2000, p62, Abcam, Cambridge, MA, USA, Mouse, 1:2000, KLF9, Abcam, Cambridge, MA, USA, Mouse, 1:1000, VEFGA, Abcam, Cambridge, MA, USA, Mouse, 1:2000, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Proteintech, Rosemont, IL, USA, 1:5000) at 4°C. Later, the membrane was incubated with secondary antibody (goat anti-rabbit IgG antibody, Yifei Xue, Nanjing, China, 1:2000) at room temperature. At last, the enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to expose the target protein. With GAPDH as an internal reference, the gray value was scanned, and the relative protein expression was analyzed.

Detection of Luciferase Activity

293T cells (Cell Culture Center, Shanghai, China) were removed after transfection and cultured for two days. The culture medium was removed, and freshly prepared double-distilled water and 5 × lysis buffer (PLB, Thermo Fisher Scientific, Waltham, MA, USA) were added and mixed well. Then, a sufficient amount of 1 × PLB was added, gently shaken at room temperature for 15min, and placed on ice for measurement. Luciferase Assay Substrate lyophilized powder (Thermo Fisher Scientific, Waltham, MA, USA) was dissolved with Luciferase Assay Buffer II (LARII). LARII 100PL was added to the detection tube to set up a fluorometer program, and 20 μL of the lysate to be tested was used to detect firefly luciferase activity. Finally, Stop & Glo. Reagent 100PL was added to the test tube to detect *Renilla* Luciferase activity, and the *Renilla* Luciferase activity value (not affected by experimental conditions) was used as an internal reference to obtain the relative activity level of firefly Luciferase.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 25.0 statistical software (IBM, Armonk, NY, USA). Data were expressed as mean ± standard deviation. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicates statistical difference. GraphPad 8.0 software was used for plotting.

Results

SGLT-2 Inhibitors Improved Cardiac Function In Diabetic Rats

After 10 weeks of SGLT-2 inhibitor intervention, the echocardiography, biochemical and pathological indicators of the rats in each group were compared to evaluate the intervention effect and safety of SGLT-2 inhibitors in diabetic myocardial disease. Compared with the NC group, fasting blood glucose in the DM group was significantly increased, triglyceride and total cholesterol levels were markedly increased, but no difference was found in liver and kidney function. Compared with the DM group, the DM + SGLT-2 inhibitor group had significantly lower fasting blood glucose. At the same time, the triglycerides and total cholesterol levels were also reduced. There was no difference in liver and kidney function among the three groups (Figure 1A-1D). It indicated that the intervention dose of SGLT-2 inhibitors in this study did not damage the liver and kidney function of SD rats. At the same time, E/A value is an index reflecting ventricular diastolic function. E/A value was markedly decreased in the DM group, indicating that left ventricular diastolic function was impaired in the DM group. The E/A of the DM + SGLT-2 inhibitor group rebounded, and it was markedly different from that in the DM group (Figure 1E). EF and FS are indexes for evaluating cardiac contractile function. Compared with NC group, LVEF and LVFS in DM group were evidently

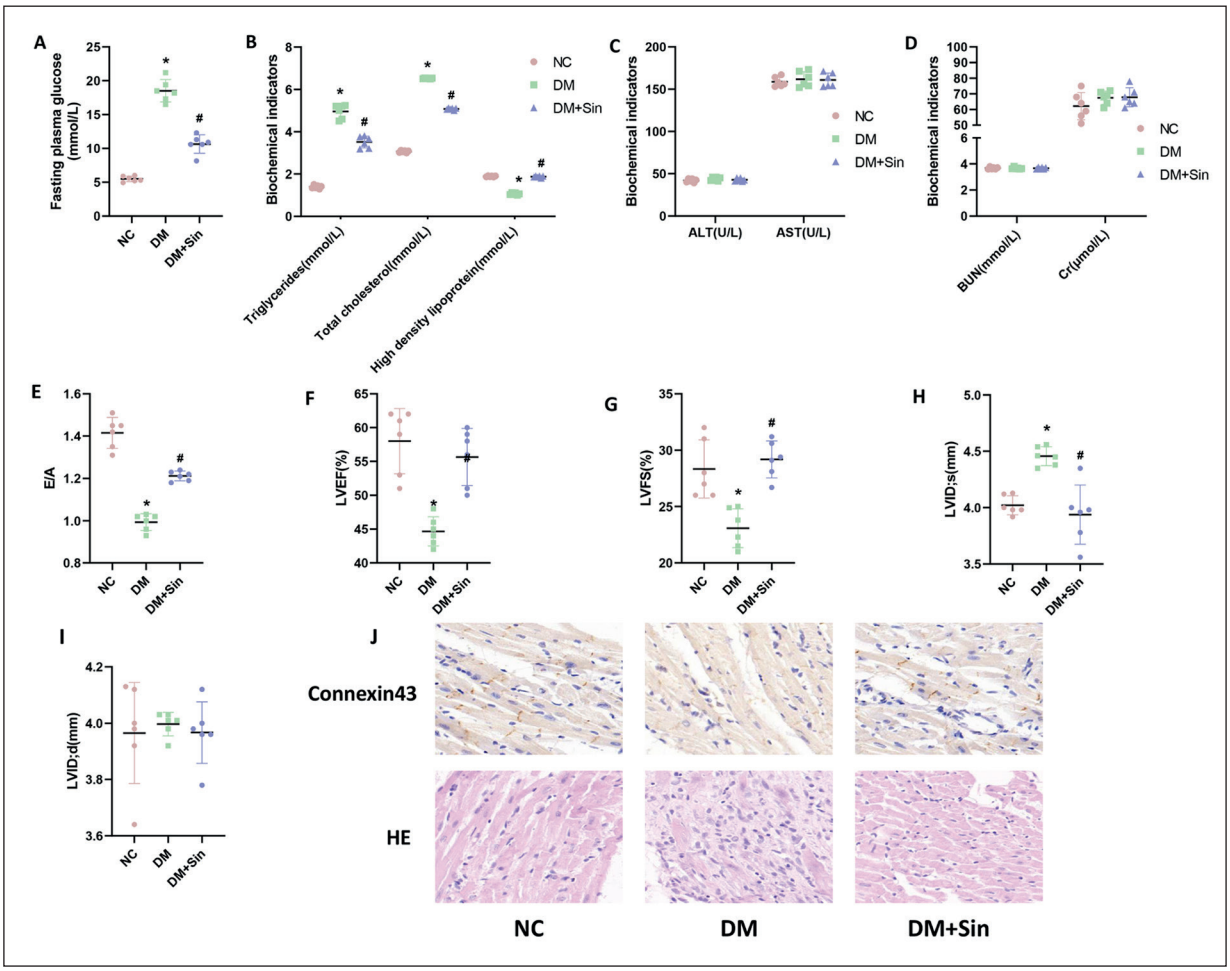

Figure 1. SGLT-2 inhibitors improved cardiac function in diabetic rats. **A-D,** The biochemical indicators of rats in NC, DM and DM+SGLT-2 inhibitor (Sin) groups ("*" indicated that compared with the NC group, $p < 0.05$). **E-I,** Echocardiography results (E/A, LVEF%, LVFS%, LVIDs, LVIDd) in NC, DM and DM+ Sin groups ("*" indicated that compared with the NC group, $p < 0.05$). **J,** HE staining of heart tissues (magnification: 400×) and Immunohistochemical staining of connexin43 in each group (magnification: 400×).

decreased. Compared with DM group, LVEF and FS in DM + SGLT-2 inhibitor group were significantly increased (Figure 1F and 1G). The LVIDs in the DM group was significantly higher than that in the NC group, indicating that the left ventricular wall was thickened, while the LVIDs in the DM + SGLT-2 inhibitor group was lower than that in the DM group, indicating that the left ventricular wall thickness was reduced (Figure 1H and 1I). The results of HE staining showed that the cardiomyocytes in the NC group were neatly arranged, the nucleus size was uniform, the cytoplasm staining was uniform, and the structure was clear. The cardiomyocytes in the DM group were hypertrophic, swollen and irregular, disorderly arranged, the nucleus size was irregular, the interstitial and extracellular matrix of myocardial cells was increased, and fibroblasts were increased. Compared with the DM group, the cardiomyocytes in the DM + SGLT-2 inhibitor group were arranged more regularly, the cell hypertrophy and edema were reduced, the muscle fibrosis was lighter, and the fibrous tissue proliferation was markedly reduced. Meanwhile, the results of immunohistochemical staining showed that connexin43 in the NC group was regularly arranged, rich in expression, evenly distributed, and located at the junction between cells. The structure of myocardial fibers was clear and aligned. The brown positive areas in the DM group were markedly reduced and arranged in disorder. Some cells lost the expression of connexin43. This showed that connexin43 expression was reduced in DCM. In contrast, the positive

area of the DM + SGLT-2 inhibitor group was increased, and connexin43 was more accurately localized, but it has not reached the normal level (Figure 1J). Therefore, the above results indicated that SGLT-2 inhibitor could improve cardiac function in diabetic rats.

Inhibition of MiR-30d Prevented Heart Function Decline In Diabetic Rats

qRT-PCR experiments were performed in rat NC group and DM group to verify the changes of miR-30-d in the heart of diabetic rats. The results found that miR-30d expression in diabetic rats was significantly higher than that in NC group (Figure 2A). To investigate the role of miR-30d in the heart of diabetic rats, the rats were divided into 5 groups: NC, NC + antagomir-NC, NC + antagomiR-30d, DM, DM + antagomiR-30d. At the end of the experiment, all groups of rats were evaluated for cardiac function by echocardiography. There was no significant difference in cardiac function among NC + antagomir-NC group, NC group, and NC + antagomiR-30d group. The E/A value, LVIDs and LVIDd of the DM + antagomiR-30d group were improved compared with the DM group, and

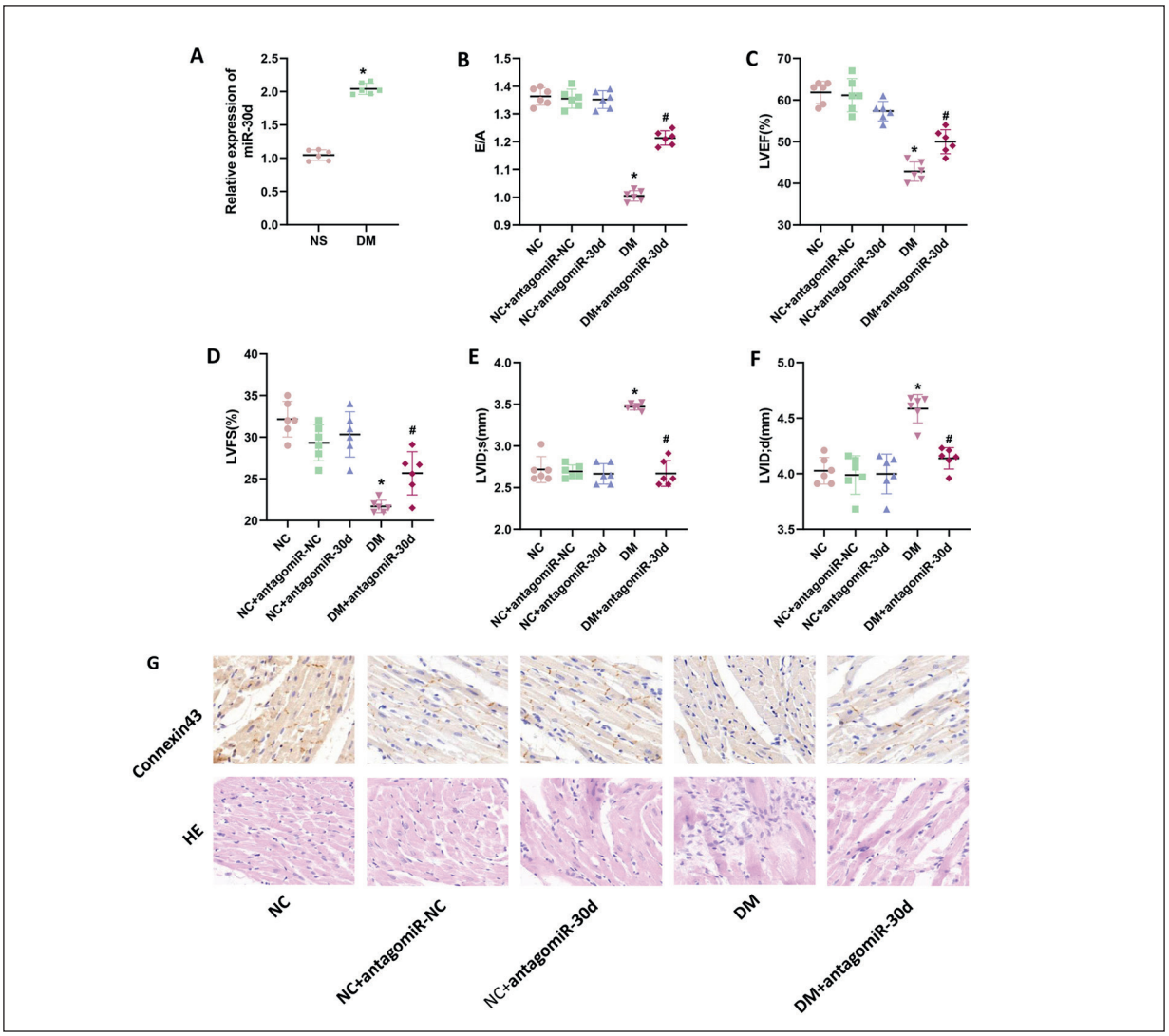

Figure 2. Inhibition of miR-30d prevented heart function decline in diabetic rats. **A,** The expression levels of miR-30d in heart tissues of NC and DM groups ("*" indicated that compared with the NC group, $p < 0.05$). **B-F,** Echocardiography results (E/A, LVEF%, LVFS%, LVIDs, LVIDd) in NC, NC+antagomir-NC, NC+antagomir-30d, DM, DM+ NC+antagomir-30d groups ("*" indicated that compared with the NC group, "#" indicated that compared with the DM group, $p < 0.05$). **G,** HE staining of heart tissues (magnification: 400×) and Immunohistochemical staining of connexin43 in each group (magnification: 400×).

there were statistical differences. This indicated that DM + antagomiR-30d group had improved diastolic function and reduced left ventricular wall hypertrophy compared with DM group. In addition, the DM + antagomiR-30d group had markedly higher LVEF and LVFS than the DM group, indicating an improvement in cardiac systolic function (Figure 2B-2F). The results of HE staining indicated that the cardiomyocytes of the NC group and the NC + antagomir-NC, NC + antagomiR-30d group were neatly arranged, the nucleus size was uniform and centered, and the cytoplasm staining was uniform. However, the cells in the DM group were disorderly arranged, myocardial cell hypertrophy was increased evidently, and fibroblasts were increased, while they were improved in DM + antagomiR-30d group, with significantly reduced fibrous tissue, but the cardiomyocytes were still hypertrophic and arranged clearly. Immunohistochemical staining was used to detect the expression of connexin43 in each group of rats. It was found that the NC group, NC + antagomir-NC, NC + antagomiR-30d had more linear yellow stains and were distributed at the junctions between the cells. The yellow stain of connexin43 was markedly reduced in the DM group compared with the NC group. On the contrary, the yellow stain of the DM + antagomiR-30d group was slightly more than that of the DM group. It was suggested that miR-30d might participate in the distribution of connexin43 and improve cardiac function (Figure 2G).

SGLT-2 Inhibitors Prevented Heart Function Decline In Diabetic Rats By Inhibiting MiR-30d

After 12 weeks of agomiR-30d injection in the tail vein and SGLT-2 inhibitor intervention, the cardiac ultrasonography and pathological parameters of each group were compared, and the intervention effect of knockdown miR-30d on DCM was evaluated. The expression levels of miR-30d in each group were detected by PCR. It was detected that DM + SGLT-2 inhibitor was down-regulated compared with DM group, while miR-30d expression of DM + SGLT-2 inhibitor + agomiR-30d was up-regulated compared with DM + SGLT-2 inhibitor + agomir-NC group (Figure 3A). At the end of the experiment, all groups of rats were evaluated for cardiac function by echocardiography. Cardiac function indexes of rats in DM + SGLT-2 inhibitor group were all recovered compared with DM group. However, the heart function of DM + SGLT-2 inhibitor + agomiR-30d group rats was worse than that of DM + SGLT-2 inhibitor group, that is, after miR-30d overexpression, the heart function of rats covered the positive regulation of SGLT-2 inhibitor intervention (Figure 3B-3F). HE staining showed that compared with DM + SGLT-2 inhibitor + agomir-NC, the DM + SGLT-2 inhibitor + agomiR-30d group had disordered cell arrangement, cardiomyocyte hypertrophy, increased fibrocytes, and significantly reduced connexin43 expression (Figure 3G). The above results indicated that SGLT-2 inhibitor prevented the decline of heart function in diabetic rats by inhibiting miR-30d.

SGLT-2 Inhibitor Can Regulate Autophagy Through MiR-30d/KLF9/VEGFA

First, autophagy-related proteins LC3-II and P62 were examined, and it was found that the autophagy level in the DM + SGLT-2 inhibitor + agomiR-30d group was lower than that in the DM + SGLT-2 inhibitor + agomir-NC (Figure 4A). Predicted by the targetscan website, it was found that miR-30d had a binding site with KLF9, which was confirmed by a Luciferase reporter gene (Figure 4B). WB verification found that miR-30d could down-regulate KLF9 expression (Figure 4C). At the same time, KLF9 regulated the expression of VEGFA gene (Figure 4D). The above results verified that the autophagy level of the rats in the DM + SGLT-2 inhibitor + agomiR-30d group was decreased compared with the rats given only the SGLT-2 inhibitor intervention, and that miR-30d could regulate the expression of KLF9 / VEGFA pathway.

Discussion

People with diabetes have a high risk of CVD, and cardiomyopathy has been identified in patients with T1DM and T2DM, which is characterized by cardiac dysfunction and oxidative stress. The occurrence of DCM is a unique pathological process that does not depend on diseases. The pathogenesis of DCM is complex with many onset factors. Impaired insulin signaling, reduced glucose transporter expression resulting in reduced glucose uptake, and increased dependence of diabetic heart on fatty acid oxidation reduce cardiac efficiency[25]. Although increasing interest concerns now the pathophysiology of DCM, there

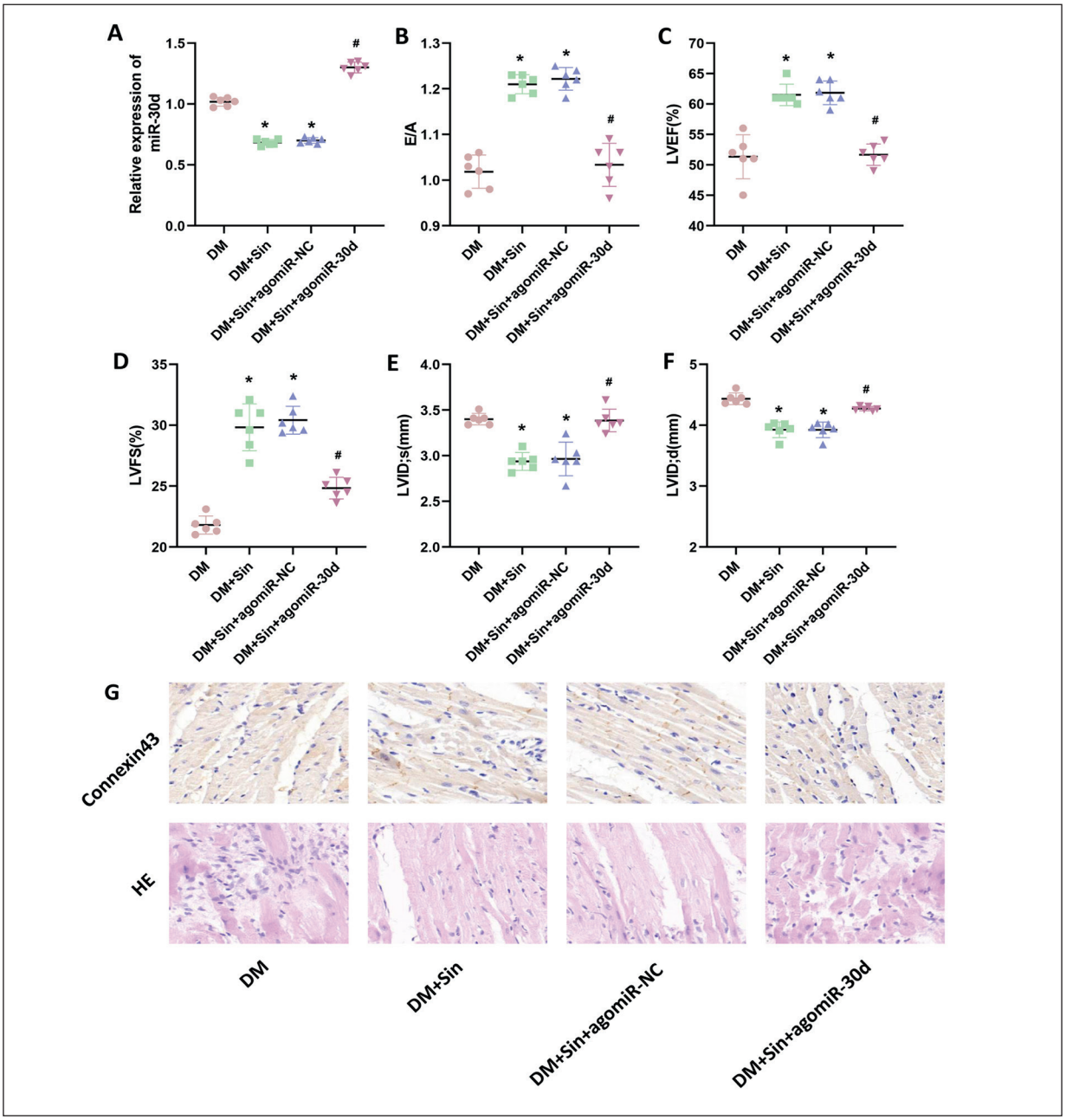

Figure 3. SGLT-2 inhibitors prevented heart function decline in diabetic rats by inhibiting miR-30d. **A,** The expression levels of miR-30d in heart tissues of DM, DM+Sin, DM+Sin+agomiR-NC, DM+Sin+agomiR-30d ("*" showed that compared with the DM group, "#" revealed that compared with the DM+Sin+agomiR-NC group, $p < 0.05$). **B-F,** Echocardiography results (E/A, LVEF%, LVFS%, LVIDs, LVIDd) in DM, DM+Sin, DM+Sin+agomiR-NC, DM+Sin+agomiR-30d groups ("*" indicated that compared with the DM group, "#" indicates that compared with the DM+Sin+agomiR-NC group, $p < 0.05$). **G,** HE staining of heart tissues (magnification: 400×) and Immunohistochemical staining of connexin43 in each group (magnification: 400×).

is no specific guidance in clinical practice for diagnosis or construction of a therapeutic strategy so far.

Autophagy refers to the lysosomal-dependent degradation mechanism of intracellular components, a cell management process that is essential for removing damaged or unwanted organelles, proteins and lipid complexes. Autophagy is a dynamic process that is tightly controlled by the availability of nutrients and cellular metabolic

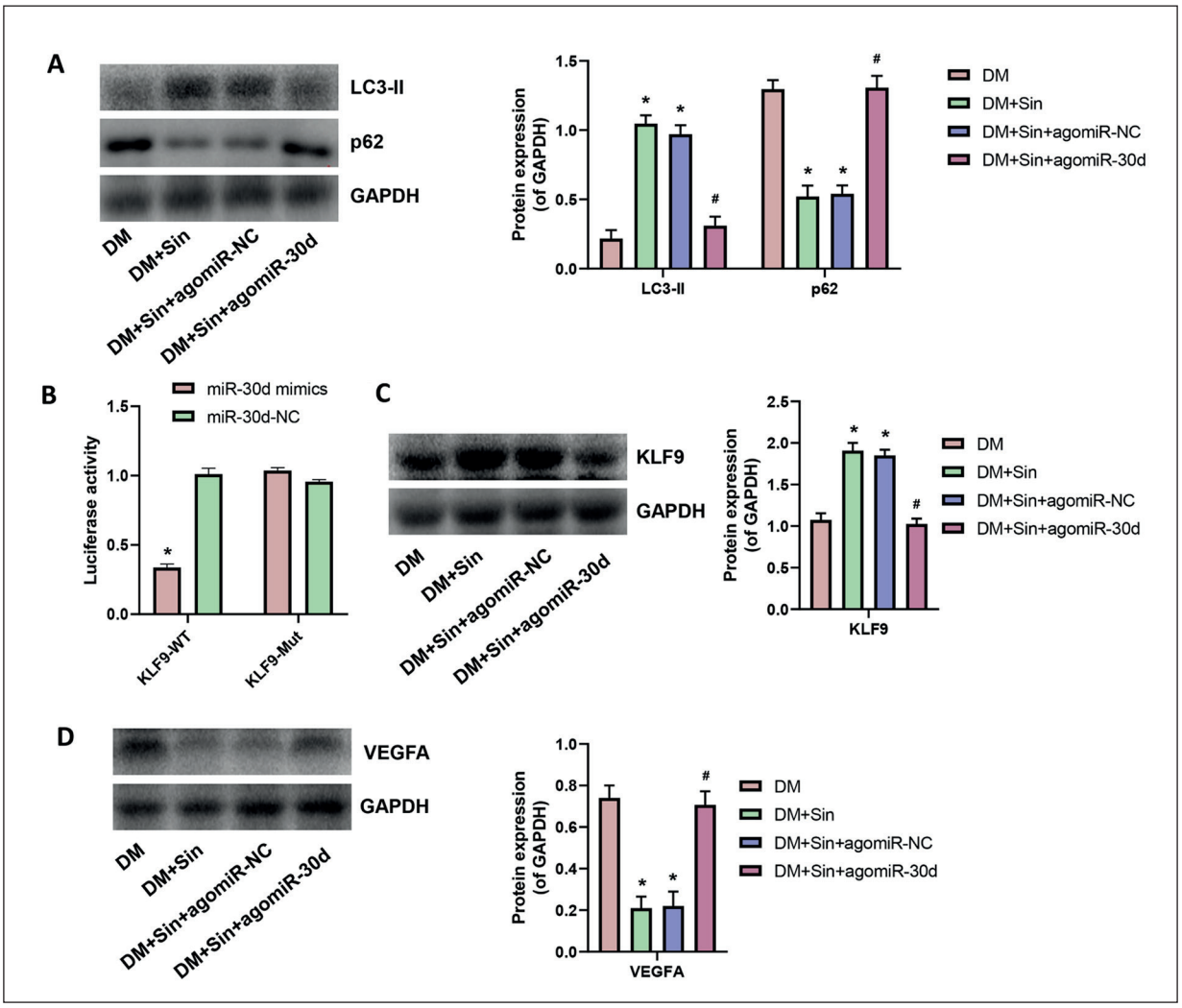

Figure 4. SGLT-2 inhibitors could regulate autophagy through miR-30d / KLF9 / VEGFA. **A,** Western blot bands and gray value analysis of LC3-II and P62 ("*" indicated that compared with the DM group, "#" indicated that compared with the DM+Sin+agomiR-NC group, $p < 0.05$). **B,** Luciferase activity ("*" revealed that compared with miR-30d mimics+ KLF9-WT group). **C,** Western blot bands and gray value analysis of KLF9 ("*" indicated that compared with the DM group, "#" indicated that compared with the DM+Sin+agomiR-NC group, $p < 0.05$). **D,** Western blot bands and gray value analysis of VEGFA ("*" showed that compared with the DM group, "#" indicated that compared with the DM+Sin+agomiR-NC group, $p < 0.05$).

balance. In the heart, autophagy is a homeostatic mechanism that maintains the structure and function of the heart, and the destruction of autophagy can lead to HF[26].

The most common method to determine autophagy activity is to detect the transformation of endogenous microtubulin 1 light chain 3I (LC3I) to microtubulin 1 light chain 3II (LC3II) by WB[27]. LC3 is the earliest autophagy marker. Its precursor was processed and the carboxyl terminal was excised to produce LC3I, which was then covalently combined with phospholipid on autophagosome membrane by ubiquitin-like modification to form LC3II[28]. The amount of LC3II is closely related to the number of autophagosomes, which is a key indicator of autophagy activity. Autophagy receptor protein P62 can be encapsulated into autophagosomes for degradation as a ubiquitination substrate and a binding factor between LC3 located on the autophagosome membrane. The expression level of P62 protein is inversely proportional to autophagy activity, which is an auxiliary indicator to detect autophagy activity[29].

In basic experiments on diabetes research, inconsistent findings have been reported on the myocardial cell autophagy up and down regula-

tion trend. In an investigation describing type 2 diabetic rodents (fructose diet model), autophagy was found to be upregulated[30]. However, experiments with a mouse model of type 1 diabetes (by injection of STZ) that destroy pancreatic β-cells have also reported a downregulation of autophagy. To support that autophagy plays a beneficial role in diabetic heart, studies have shown that activation of autophagy by fenofibrate metformin or resveratrol can improve cardiac dysfunction caused by diabetes[31-33]. Chloroquine can exacerbate diastolic dysfunction caused by diabetes by inhibiting autophagy. In contrast, other researchers suggest that autophagy may play an adverse role in diabetic heart. Therefore, whether upregulation of autophagy is protective or harmful to the heart is a controversial issue. However, according to the results of this study, the recovery of autophagy level caused by miR-30d knockdown was beneficial in hindering the occurrence of DCM.

The KLF family is composed of 17 members with diverse regulatory functions[34]. The Krüppel-like factor 9 (KLF9) is a member of the KLFs family and is also known as BTE-B1 (basic transcription element-binding protein 1). In the current study, KLF9 was negatively correlated with miR-30d expression. The Luciferase reporter assay confirmed that KLF9 was a target gene of miR-30d. The attenuated expression of KLF9 regulated cardiomyocytes autophagy. Ma et al[35] have shown that KLF9 can directly bind to the promoter of VEGFA, thereby regulating gene expression. The VEGF family includes VEGFA, VEGFB, VEGFC, and VEGFD. Among them, VEGFA is the most commonly used one and can promote neovascularization[36]. Kang et al[37] have shown that VEGF can inhibit excessive autophagy induced by chronic cerebral ischemia, thereby protecting cognitive function. Domigan and Iruela-Arispe[38] found that depletion of endothelial cell autocrine VEGF can cause mitochondrial disruption, inhibition of glucose metabolism, enhanced autophagy, and eventually cell death.

Considering the increasing threat of DCM to T2DM patients, more specific studies should be conducted to better understand the effects of SGLT-2 inhibitors on diastolic and systolic dysfunction. Revealing the mechanism is very important for clinical prevention and treatment of DCM. In this study, differentially expressed miR-30d was found from the perspective of DM and DM+ SGLT-2 inhibitor animal model, and its regulatory mechanism of participating in the autophagy induced by hyperglycemia by inhibiting its target gene KLF9 was revealed, providing new clues for the mechanism research and clinical treatment of SGLT-2inhibitor regulating DCM.

Conclusions

In summary, it can be concluded that SGLT-2 inhibitor can promote the autophagy of cardiomyocytes by inhibiting the expression of miRNA-30d, which can negatively regulate its target gene KLF9, thus improving the DCM rats' cardiac function.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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