

Sitagliptin inhibits EndMT *in vitro* and improves cardiac function of diabetic rats through the SDF-1 α /PKA pathway

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Abstract. – OBJECTIVE: The aim of this paper was to study sitagliptin in improving the endothelial-mesenchymal transition (EndMT) of human aortic endothelial cells (HAECs) and cardiac function of rats with diabetes mellitus (DM) and its possible pathway.

MATERIALS AND METHODS: Sprague Dawley (SD) rats were divided into control group, DM group and sitagliptin group. The myocardial contraction and relaxation functions of rats in each group were observed via echocardiography. The changes in cardiac structure and fiber were observed via hematoxylin-eosin (HE) staining, Masson staining and Sirius red staining. The immunohistochemical assay was performed to observe the expressions of α -smooth muscle actin (α -SMA) and VE-cadherin in HAECs; the expression of reactive oxygen species (ROS) in HAECs was detected using the fluorescence probe. Moreover, the expressions of transforming growth factor- β 1 (TGF- β 1), phosphorylated-protein kinase A (p-PKA), PKA and extracellular signal-regulated kinase (ERK) were observed via Western blotting.

RESULTS: Sitagliptin could improve the myocardial contraction and relaxation functions in diabetic rats and EndMT and ROS production in HAECs. In the DM group, the expression of Glucagon-like peptide-1 (GLP-1) was decreased, while the expression of stromal-derived factor-1 α (SDF-1 α) was decreased and the expressions of downstream PKA/ERK pathway and TGF- β 1 were increased. The above changes could be reversed by sitagliptin.

CONCLUSIONS: Sitagliptin can reverse the EndMT in HAECs as well as the cardiac function in diabetic rats through the SDF-1 α /PKA pathway.

Key Words:

Sitagliptin, Endothelial-mesenchymal transition, Human aortic endothelial cells, Cardiac function, Diabetes mellitus.

Introduction

Diabetes mellitus (DM) is a systemic disease affecting the life quality and longevity of patients^{1,2}. Studies³ have demonstrated that there are changes in the myocardial structure in DM independent of coronary heart disease and hypertension, so diabetic cardiomyopathy has been recognized as a special diabetic complication. According to the epidemiological investigation, DM has a strong correlation with heart failure, and the possible reason is that myocardial fibrosis caused by DM reduces myocardial compliance, increases the risk of arrhythmia and affects myocardial function, ultimately leading to heart failure⁴.

Myocardial fibrosis refers to the excessive accumulation of collagen fibers in the myocardial tissue structure, the significant increase in collagen concentration or the increase in collagen volume fraction⁵. Myocardial fibrosis is accompanied by the remodeling of the myocardial interstitial network. As a result, the coexistence of fibrosis and ventricular remodeling causes severe damage to the myocardial contraction and relaxation functions, ultimately leading to refractory congestive heart failure⁶. Myocardial fibrosis is caused by degeneration, necrosis and apoptosis of myocardial cells, thereby activating macrophages to produce various cytokines and promoting the formation of the extracellular matrix (ECM) in the myocardial interstitial cells. In recent years, endothelial-mesenchymal transition (EndMT) has attracted more attention from scholars⁷. EndMT is a special form of epithelial-mesenchymal transition (EMT), which is manifested as loss of endothelial cell markers and increase of mesenchymal cell markers⁸. In

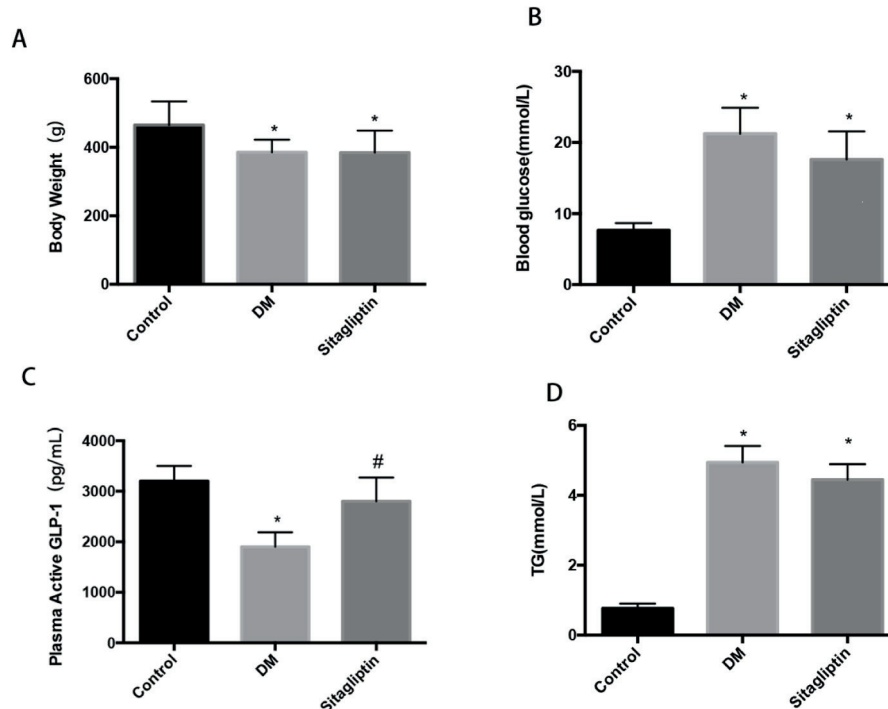


Figure 1. Body weight (A), blood glucose (B), total triglyceride (C), GLP-1 (D) in each group. # $p < 0.05$ vs. control, * $p < 0.05$ vs. DM.

diabetic cardiomyopathy, EndMT also exists as a pathway affecting myocardial function^{9,10}.

Glucagon-like peptide-1 (GLP-1), a peptide hormone secreted by intestinal L cells, can promote the insulin secretion, inhibit the glucagon secretion and keep the stability of blood glucose in a glucose-dependent manner¹¹. The secretion level and activity of GLP-1 significantly decline in patients with type 2 diabetes mellitus (DM)¹². Endogenous GLP-1 can be decomposed rapidly by dipeptidyl peptidase 4 (DPP-4). Sitagliptin, as a new DPP-4 inhibitor (DPP-4i), can inhibit the activity of DPP-4, delay the degradation of GLP-1 *in vivo* and reduce the blood glucose¹³. Moreover, it has been proved that DPP-4i, besides the hypoglycemic effect, can also resist atherosclerosis, improve ventricular function¹⁴ and fibrosis in diabetic nephropathy and reduce proteinuria production through the stromal-derived factor-1 α (SDF-1 α) pathway¹⁵. However, the effects of DPP-4i on myocardial fibrosis and EndMT in human aortic endothelial cells (HAECs) remain unclear. Therefore, whether DPP-4i can improve the myocardial fibrosis and EndMT in HAEC in DM through the SDF-1 α pathway was explored in this study.

Materials and Methods

Animals and Reagents

40 Sprague Dawley rats aged 8 weeks in normal nutritional and mental status were provided by the Laboratory Animal Center of Shandong University. This study was approved by the Animal Ethics Committee of the Shanxian Central Hospital Animal Center. Transforming growth factor- β 1 (TGF- β 1), α -smooth muscle actin (α -SMA) and VE-cadherin antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hematoxylin-eosin (HE) staining, Masson staining and Sirius red staining kits, reactive oxygen species (ROS) assay kit, enzyme-linked immunosorbent assay (ELISA) kits of triglyceride and serum GLP-1 were purchased from Beyotime (Shanghai, China). SDF-1 α , phosphorylated-protein kinase A (p-PKA), AKT, extracellular signal-regulated kinase (ERK) and DPP-4 antibodies were bought from Cell Signaling Technologies (CST; Danvers, MA, USA).

Establishment of Rat Model of DM

40 male Sprague Dawley rats aged 8 weeks were randomly divided into the control group

(n=10), the DM group (n=15) and the sitagliptin group (n=15). In the DM and sitagliptin groups, rats were fed with the high-fat diet for 4 weeks and then intraperitoneally injected with streptozotocin (STZ) (30 mg/kg). Within 1 week after intraperitoneal injection, rats whose fasting blood glucose level was lower than 11.1mmol/L were eliminated. After stable modeling for 2 weeks, rats in the sitagliptin group were intragastrically administered with sitagliptin (10 mg/kg^{*}d) till the end of the experiment. Rats in other groups were intragastrically administered with normal saline. After rats were fed with sitagliptin for 12 weeks, they were executed, the serum was retained, the heart was weighed and the heart weight/body weight ratio was calculated.

Cell Culture

Human artery endothelial cells (HAECs) were cultured in the extracellular matrix (ECM) supplemented with 5% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C. During the experiments, the culture medium was changed to a serum-free solution for 12 hours. Then, the cells were treated with 5 mmol/L glucose (control), 30 mmol/L glucose (high glucose [HG]), high glucose with 0.1 µmol/mL Sitagliptin (Sitagliptin) for 3 days. The medium was changed every 24 h. Sitagliptin was dissolved in 1% (v/v) dimethylsulfoxide (DMSO) and the DMSO group was used as a control to rule out the effect of the vehicle. Mannitol (MA) was used as a control to rule out the effect of osmotic pressure. The cells were harvested for analysis after 24 h.

Body Weight and Glucose-Lipid Metabolism in Rats in Each Group

The body weight, blood pressure and blood glucose were detected every week during the experiment. The levels of serum total triglyceride, Glucagon-like peptide-1 were detected at the end of the experiment.

Echocardiography

The transthoracic echocardiography was performed for diabetic rats using the VisualSoCVIE-VO 2100 and 35 MHz probes before injection of STZ and at 0, 4, 6, 8 and 10 weeks after injection. After the rats were anesthetized with isoflurane, the left ventricular fractional shortening (FS), ejection fraction (EF), cardiac output (CO), heart rate (HR), left ventricular internal diameter at end-diastole (LVIDd) and left ventricular internal

diameter at end-systole (LVIDs), isovolumic relaxation time (IVRT), peak velocity of early (E) and late (A) filling wave and mitral deceleration time were measured.

Pathological Staining of Myocardial Tissues

After fasting for solids not liquids overnight, rats were anesthetized and the thoracic cavity was opened to expose the heart. The heart and aorta were taken under aseptic conditions, fixed with 10% formaldehyde, dehydrated, routinely embedded in paraffin and sliced into about 5 µm-thick sections at 60°C overnight, followed by deparaffinization with xylene and dehydration with gradient alcohol. The same 4 sections were taken from the aortic root of each rat and 2 sections were taken at an interval of 100 µm, followed by HE staining, Masson staining, Sirius red staining and observation under a light microscope. Finally, the sections were analyzed using the Image-Pro Plus (IPP; Microsoft Corporation, Redmond, WA, USA).

Immunofluorescence Staining

HAECs were cultured on 12-chamber slides. The cells were fixed with 4% paraformaldehyde for 15 min and then blocked with 3-5% bovine serum albumin (BSA) for 30 min. The cells were then incubated with 4',6-diamidino-2-phenylindole (DAPI), VE-cadherin or anti-α-SMA antibody for 1 h 30 min, followed by incubation with FITC-conjugated secondary antibodies for 45 min. Finally, the cells were observed under a fluorescence microscope.

ROS Staining

HAECs were washed with phosphate-buffered saline (PBS) and then incubated with 2,7-dichlorofluorescein-diacetate (H2DCF-DA) at room temperature for half an hour. The ROS level was determined by the oxidative conversion of H2DCF-DA to fluorescent dichlorofluorescein on reaction with ROS in cells. HAECs were incubated with dihydroethidium for 30 min at room temperature. After washing with PBS, fluorescent signals (ROS, 488 nm) were captured by using a Leica microscope. Three independent experiments were conducted.

Western Blotting

After protein quantification for cell and tissue lysate, the protein was added with the loading buffer, heated and denatured, followed by sodium dodecyl sulphate-polyacrylamide gel electropho-

resis (SDS-PAGE). Then, the protein was transferred onto a membrane, sealed with 5% skim milk for 2 h and incubated with the primary antibody at 4° C overnight. After the membrane was washed with Tris-Buffered Saline with Tween-20 (TBST) 3 times (10 min/time), the protein was incubated with the corresponding secondary antibody at room temperature for 1 h. After the membrane was washed again with TBST 3 times (10 min/time), the expression of the different protein samples was detected *via* electrochemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Data were expressed as mean \pm standard deviation, and analyzed by paired or unpaired *t*-test. One-way analysis of variance was adopted among groups, and pairwise comparison was performed *via* Student-Newman-Keuls (SNK) post-hoc test. $p < 0.05$ suggested that the difference was statistically significant. Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for data analysis, and Graph-Pad software (La Jolla, CA, USA) was used for plotting.

Heart Weight, Blood Glucose and Blood Lipid in Each Group

At the end of the experiment, the body weight was decreased in the DM group compared with those in the control group, while in the sitagliptin group no significant improvement was observed. The blood glucose, total triglyceride were increased in the DM group compared with those in the control group, while the blood glucose and total triglyceride had no significant improvement in the sitagliptin group compared with those in the DM group. The GLP-1 levels in plasma were decreased in the DM group compared with those in the control group. And sitagliptin can reserve the change (Figure 1).

Sitagliptin Could Improve Myocardial Function and Fibrosis of Diabetic Rats

Before the experiment, the size of the left heart chamber, LVIDs and LVIDd had no significant differences among groups. At the end of the experiment, LVIDs and LVIDd were increased in the DM group compared with those in the control group, and they were decreased in the sitagliptin group compared with those in the DM group. In terms of left ventricular systolic function, FS and EF had no significant differences at the beginning

of the experiment among groups. At the end of the study, FS and EF were decreased in the DM group compared with those in the control group, and they were increased in the sitagliptin group compared with those in the DM group, indicating that sitagliptin can improve the left ventricular systolic function of diabetic rats. In terms of left ventricular diastolic function, the E/A ratio was reduced; IVRT and mitral deceleration time were prolonged in the DM group, while the E/A ratio was increased. IVRT and mitral deceleration time were shortened in the sitagliptin group compared with those in the DM group, indicating that sitagliptin can improve the high glucose-induced diastolic dysfunction (Table I).

In the HE stained specimens, the left ventricular dilation, ventricular wall hypertrophy, myocardial hypertrophy, disordered arrangement and rupture of the muscle fiber and irregular shape could be seen in the DM group. After sitagliptin intervention, the left ventricle shrank, hypertrophy was alleviated, myocardial cell volume was reduced and cell arrangement was ordered. Masson staining and Sirius red staining showed that the collagen was thick and disorganized with uneven distribution, and the collagen fibers deposited were increased in the DM group. In the sitagliptin group, there was less collagen deposition and its arrangement was more regular than the DM group. Both type I and type III collagen fibers were significantly increased in the DM group, while they were decreased in the sitagliptin group compared with those in the DM group. EndMT often occurs in the early stage of myocardial fibrosis. The content of TGF- β 1, an inducer of EMT, was detected in tissues. It was found that

Table I. Sitagliptin improves cardiac function in diabetic mice.

	Control	DM	Sitagliptin
LVIDd (mm)	6.56 \pm 0.24	7.33 \pm 0.26*	6.8 \pm 0.29 [#]
LVIDs (mm)	3.43 \pm 0.19	4.63 \pm 0.20*	3.63 \pm 0.28 [#]
FS (%)	42.41 \pm 2.16	35.52 \pm 2.31*	41.43 \pm 2.81 [#]
E/A	1.32 \pm 0.09	1.01 \pm 0.06*	1.27 \pm 0.13 [#]
IVRT (ms)	27.43 \pm 3.12	44.53 \pm 2.19*	30.24 \pm 3.3 [#]
EF (%)	72.43 \pm 2.98	63.32 \pm 2.30*	69.31 \pm 3.10 [#]

LVIDd (left ventricular internal diameter at end-diastole); LVIDs (left ventricular internal diameter at end-systole); FS (the left ventricular fractional shortening); E/A (E: peak velocity at early diastole. A: peak velocity at late diastole); IVRT (isovolumic relaxation time); EF (ejection fraction) of each group. Data are presented mean \pm SD; [#] $p < 0.05$ vs. control, * $p < 0.05$ vs. DM.

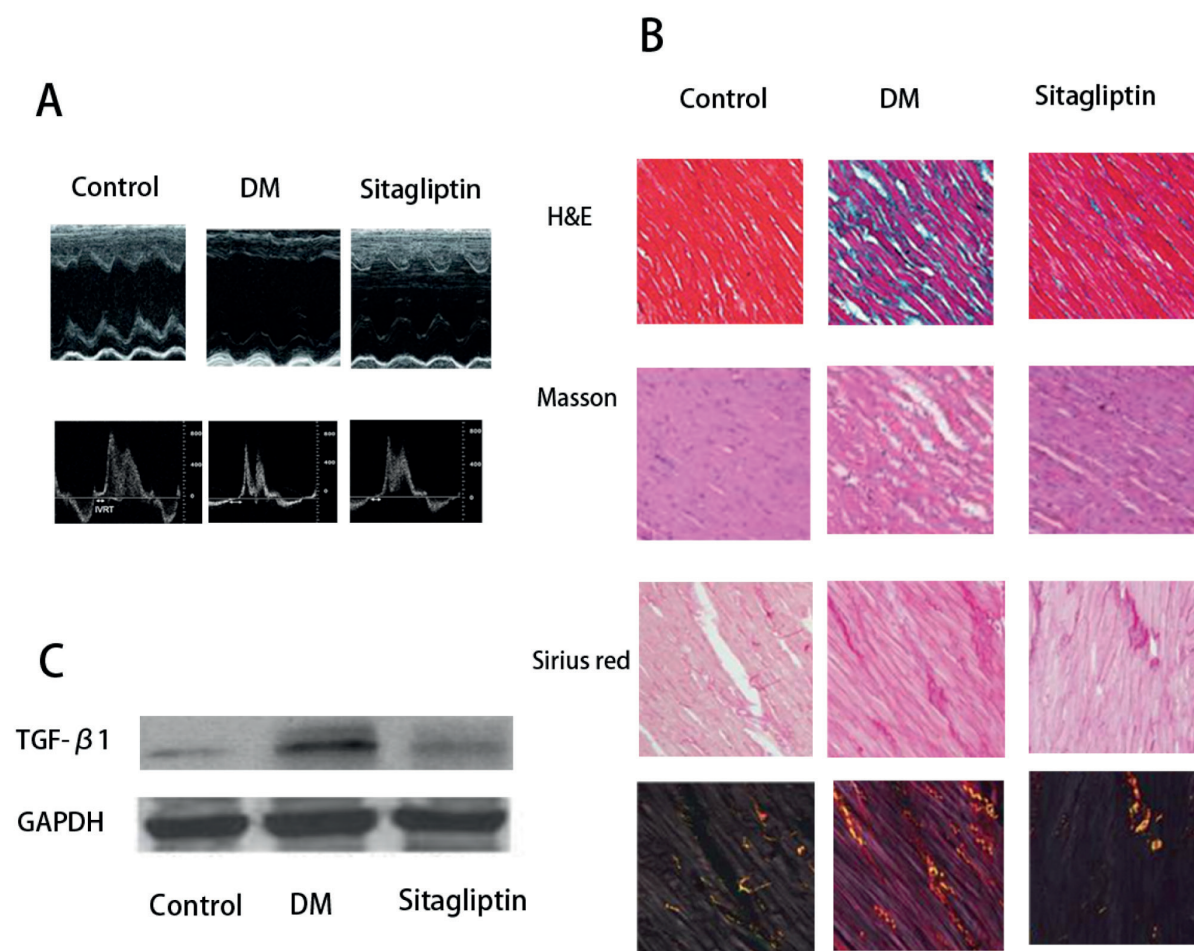


Figure 2. Sitagliptin improves the myocardial function of rats. **A**, M-mode echocardiogram of rats in each group. **B**, Doppler image of mitral flow in each group. **C**, Line 1: HE staining of myocardial sections in each group: myocardial hypertrophy and irregular morphology of muscle fiber can be observed in the DM group, and the symptoms are improved in the sitagliptin group compared with the DM group. Line 2: Masson staining of myocardial sections in each group: there is significant hyperplasia of collagen fibers in the DM group, and decline in collagen deposition in the sitagliptin group. Line 3-4: Sirius red staining of myocardial sections in each group: type I and type III collagen fibers are significantly increased in the DM group, while they are decreased in the sitagliptin group compared with those in the DM group. **D**, TGF-β1 protein content in myocardial cells in each group.

TGF-β1 was significantly increased in the DM group and decreased in the sitagliptin group compared with that in the DM group, suggesting that sitagliptin can improve the DM-induced fibrosis through TGF-β1 (Figure 2).

Sitagliptin Improved EndMT in HAECs

The expression of α-SMA, the marker for mesenchymal cells, was up-regulated in the DM group and down-regulated in the sitagliptin group. The expression of VE-cadherin, the marker for endothelial cells, was down-regulated in the DM group and up-regulated in the sitagliptin group. In myocardial cells of rats, the protein content of α-SMA was increased in the DM group and decreased in

the sitagliptin group. The protein content of vimentin was the opposite, which were consistent with the results in the cell experiment, indicating that sitagliptin can improve the DM-induced EndMT in HAECs and increase the content of myocardial fibroblasts (Figure 3).

Sitagliptin Increase the Expression of SDF-1α, Affected the Downstream PKA/ERK Pathway of SDF-1α and Improved the Oxidative Stress of HAECs

DPP-4 removes GLP-1, BNP and SDF-1α in the body, which explains the protective effect of DPP-4i on tissue and organ besides the hypoglycemic effect. SDF-1α, also known as CXCL12, is a sub-

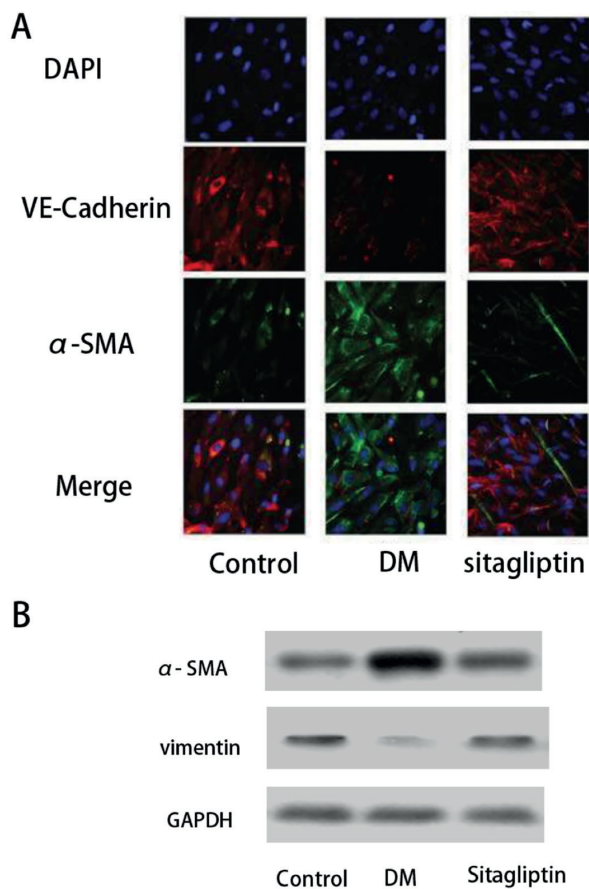


Figure 3. Sitagliptin improves the EndMT in HAECs. **A**, VE-cadherin (red), α -SMA (green) and DAPI (blue) staining in HAECs. **B**, Protein content of α -SMA and vimentin in myocardial cells of rats.

strate for DPP-4, which has been proved to possess the cardioprotective effect in recent studies. It has also been proved that DPP-4i can improve renal fibrosis through the SDF-1 α pathway in the kidney. The expression of SDF-1 α was detected in *in vitro* experiments, and it was found that the SDF-1 α level was reduced in the DM group and increased in the sitagliptin group.

PKA is a downstream pathway of SDF-1 α , so the p-PKA level was detected. The p-PKA level significantly declined in the DM group, and in the sitagliptin group it was similar to that in the control group. The activation of PKA could inhibit NADPH oxidase, thus reducing the production of oxidative stress and the activation of downstream ERK pathway, ultimately affecting the expression of TGF- β 1. In *in vitro* experiments, the ROS production, p-ERK expression and TGF- β 1 expression were increased in the DM group, and they were decreased in the sitagliptin group. The

above findings are consistent with our suggestion (Figure 4).

Discussion

Diabetic cardiomyopathy is characterized by ventricular dysfunction, which is manifested as the early diastolic dysfunction in DM patients without coronary artery disease or hypertension¹⁶. In this experiment, both myocardial diastolic and systolic dysfunction could be observed in diabetic rats. The pathological feature of diabetic cardiomyopathy is myocardial fibrosis, which is mediated by myocardial fibroblasts and also involves macrophages, myocardial cells and vascular cells¹⁷.

Fibroblasts are involved in tissue repair in the normal physiological process, which are the main source of ECM¹⁸. Fibroblasts are also involved in the pathological process of myocardial fibrosis. Most of the cardiac fibroblasts come from embryo mesenchyma stem cells in the process of myocardial repair, and recent studies¹⁹ have found that endothelial cells can also be transformed into fibroblasts through EndMT, thus participating in tissue repair. EndMT is a common physiological process of tissue development during cardiac development in the early embryonic stage. The endocardium forms interstitial cells through EMT, further forming the atrioventricular cushion, primitive valve and cardiac septum, which is regulated by TGF- β 1 and BMPs²⁰. In adulthood, the abnormal EndMT and the resulting myofibroblasts and fibrocytes play important roles in tissue fibrosis²¹. In this experiment, it was observed that the expression of TGF- β 1, an EndMT-promoting factor, was increased. In *in vitro* experiments, the expression of fibroblast markers was increased, while the markers for endothelial cells were decreased, indicating that EndMT is activated and sitagliptin can reverse these changes.

In existing experiments, SDF-1 α remarkably declines under high-glucose environment. The possible reason is that the DPP-4 activity is increased and SDF-1 α clearance is faster in high-glucose environment²². In this work, after treatment with sitagliptin, the DPP-4 expression was reduced, while the expressions of SDF-1 α and PKA pathway were increased. SDF-1 α can activate the PKA pathway through the G protein-coupled receptor, thereby reducing the NOX2 formation and ROS production²³. ROS can regulate the TGF- β 1-mediated EMT²⁴. In this study, the p-PKA expression was increased, while the expressions of ROS and p-ERK were decreased in

the sitagliptin group, suggesting that the oxidative stress increases in a high-glucose environment and the expression of TGF- β 1 also increases, while sitagliptin can reverse these changes, reduce oxidative stress and lower the expression of the EndMT-promoting factor. EndMT frequently occurs in the early stage of myocardial fibrosis, which is a switch for fibrosis and a potential therapeutic target for fibrosis²⁵.

function *in vivo* and reduce the EndMT in HAECs through the SDF-1 α /PKA pathway, which also provides new ideas for the prevention and treatment of diabetic cardiomyopathy in the future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Conclusions

We showed that sitagliptin can improve the DM-induced myocardial fibrosis as well as cardiac

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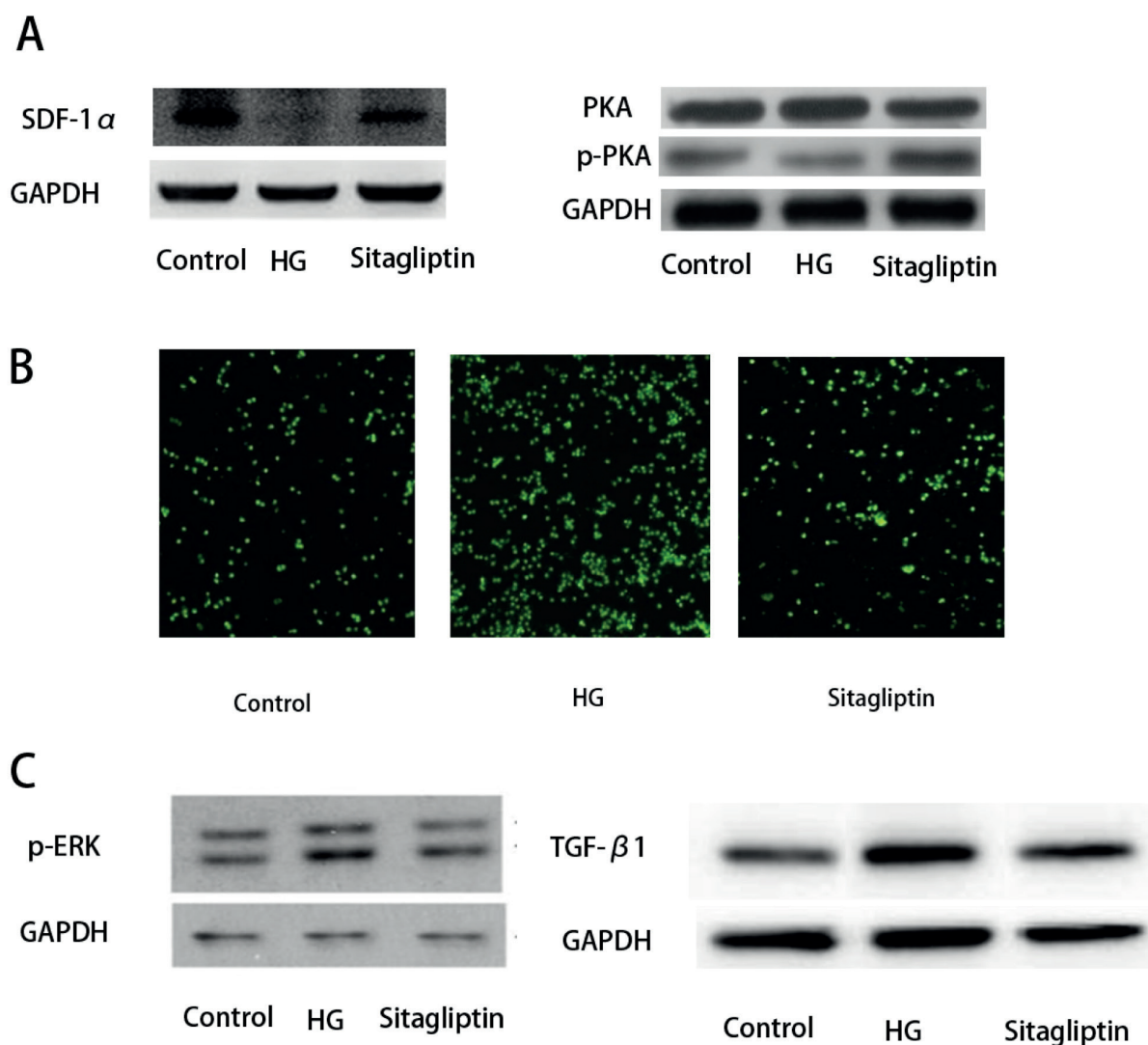


Figure 4. *A*, Protein expression of SDF-1 α and p-PKA in HAECs. *B*, ROS expression in HAECs. *C*, Expression of ERK and TGF- β 1 in HAECs in each group.

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