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Liraglutide alleviates vascular injury in diabetic rabbits with lower limb vascular stenosis through regulation of RCAN1

H.-X. DING[1], N.-X. DONG[1], C.-X. ZHOU[2], F.-J. WANG[1], N. XING[1], H.-F. MA[1], L. HOU[1]

[1]Department of Endocrinology, [2]The Second Department of Surgery, The Fourth Hospital of Hebei Medical University, Shijiazhuang, China

Abstract. – OBJECTIVE: The aim of the present study is to explore the possible mechanism that may have ameliorative effect of liraglutide (Lira) on diabetic lower extremity vascular stenosis.

MATERIALS AND METHODS: A diabetic rabbit model of lower extremity stenosis was established and treated with Lira. The intimal hyperplasia of the lower extremity and the oxidative stress level of vascular tissue were observed and examined. Vascular smooth muscle cells (VSMCs) induced by high glucose (HG) were treated with Lira, and RCAN1 overexpressing plasmid was constructed to transfect VCMCs.

RESULTS: Lira treatment showed its association in significantly improving the hyperplasia of the intima, the level of oxidative stress, and the level of homeostasis model assessment of insulin resistance (HOMA-IR) in rabbits induced by diabetes and lower limb stenosis. In addition, Lira treatment reduced the elevated expression of RCAN1 in vascular tissues induced by diabetes. Not only could Lira treatment inhibit the increase of ROS level, proliferation and migration of VSMCs induced by HG, but reduce the expression of PCNA, MMP-9 and collagen I induced by HG. Overexpression of RCAN1 in VSMCs counteracted the effect of Lira, suggesting that Lira affected the proliferation and migration of VSMCs by regulating RCAN1.

CONCLUSIONS: Our findings have important implications for Lira to exert beneficial outcomes in reducing excessive neointimal formation after lower extremity vascular injury in diabetic rabbits *via* the regulation of RCAN1.

Key Words:
Liraglutide, Diabetes mellitus, Arterial occlusive disease, Diabetic rabbit model, RCAN1, Vascular smooth muscle cells.

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia. Copious studies showed DM to be a global public health burden. International Diabetes Federation (IDF) reported a high prevalence of DM: approximately 415 million adults between the ages of 20 to 79 years in 2015 and with a trend towards gradual increase in having another 200 million by 2040[1]. Diabetic foot is a substantial chronic problem in DM. Epidemiological data have showed that lower extremity peripheral artery disease (PAD) affects 12% to 20% of Americans over 60 years old[2]. Moreover, PAD affects 15% of the Australian population and is a powerful and serious predictor of cardiovascular mortality[3]. The complexity of diabetic foot mainly includes ischemic neuropathy, wound healing infection, and so on. The diabetic foot does not only increase vascular disease but is a consequence of severe limb ischemia.

Currently, both type 1 and type 2 diabetes can be treated intravascularly, but only for macrovascular complications, not microvascular ones. Endothelial regeneration is an important process of vascular injury after interventional therapy, and hyperglycemia in diabetes can impair endodermal integrity, leading to delayed endothelial regeneration and excessive formation of new intima[4]. However, giving the increased risk of amputation and mortality, debates exist about the application of paclitaxel coating for most actual endovascular treatments (namely drug-eluting balloons and stents).

Liraglutide is one of the glucagon-like peptide-1 (GLP-1) receptor agonists that stimulates glucose-dependent insulin secretion, inhibits glucagon release, and reduces food intake, thereby improving blood sugar and weight loss in patients with diabetes[5]. Studies[6,7] have shown that liraglutide plays an important role in vascular damage in diabetes by its influence on glucose and lipid metabolism and the activation of adhesion molecules. It has been proposed that liraglutide could

Corresponding Author: Hai-Xia Ding, MD; e-mail: dhx-369@163.com

limit vascular injury in diabetes by activating GLP-1 receptors and inhibiting abnormal cell migration, proliferation and apoptosis induced by high glucose in vascular smooth muscle cells[8]. Regulator of calcineurin 1 (RCAN1) is a stress-inducible protein whose main functions include the inhibition of phosphatase calcineurin and regulation of mitochondrial function in response to cellular stress, such as reactive oxygen species and hyperglycemia[9]. It was found that overexpression of RCAN1 resulted in a decrease in glucose-stimulated insulin secretion (GSIS) in β cells and in the number and quality of mouse β cells[10]. However, there is limited data on the role of RCAN1 in vascular injury in diabetes. This study aims to investigate whether the therapeutic effect of liraglutide on lower limb vascular injury in diabetic patients is related to RCAN1.

Materials and Methods

Animal Model Establishment

The lower limb ischemia model of diabetic rabbits was established as follows: rabbits weighing 2.5-3.0 kg were fed with the high-fat diet for 2 months, intraperitoneally injected with 0.05 mmol/L streptozotocin as 60 mg/kg. Rabbits with fasting blood glucose over 16.7 mmol/L were selected to establish a model with lower limb ischemia. The study protocol was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University.

The diabetic rabbits were divided randomly into 2 groups (6 rabbits in each group): ischemic model (DM) and liraglutide treated ischemic group (DM+Lira). All rabbits were anesthetized by intraperitoneal injection of 3% pentobarbital sodium (1 mL/kg). A minimum of 1 cm of the left femoral artery was carefully exposed and separated from the surrounding tissue after a 2.5 cm incision from the middle of the right inguinal to the inside knee was made. Then the separated femoral artery was ligated in the ischemic model. The incision was sutured, and gentamicin was used to prevent infection.

The drug treatment for 4 weeks started on the first day after the ischemic injury with a dose of 200 μg/kg/d, and the rabbits in the normal and untreated diabetic ischemic injury group were given an equal volume of saline solution. Rabbits without any treatment were allocated into the control group (CTRL).

The experiment animals were routinely observed once a day after intervention. An invasive procedure was performed to reduce the pain of the animals using an intraperitoneal injection of 10% chloral hydrate 5 ml of anesthesia. The rabbits were killed by carbon dioxide inhalation after 4 weeks.

Staining of the Femoral Artery

After fixation with paraformaldehyde and decalcified in 10% EDTA, the tissues were embedded in paraffin and sectioned into a 5-μm-thick section. The selected sections were deparaffinized in xylene and were rehydrated through a graded series of ethanol washes followed by hematoxylin and eosin (H&E) staining (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's manual. The cross-sectional area of media and intima was measured using Image J software (NIH, Littleton, CO, USA) and the intima/media ratios were calculated.

Detection of MDA, SOD, and HOMA-IR in Arterial Tissues

The concentration of malondialdehyde (MDA) was determined to assess the level of lipid peroxidation. The level of superoxide dismutase (SOD) activity was measured to evaluate the antioxidant levels. We also calculated homeostasis model assessment of insulin resistance (HOMA-IR) as fasting blood glucose (FBG) (mmol/L) × fasting insulin (FIN) (mU/L)/22.5. Commercial assay kits in our study were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and conducted in accordance with the manufacturer's instructions.

VSMCs Culture and Treatment

Human Vascular Smooth Muscle Cells (VSMCs) were purchased from Cell Application at the forth passage, cultured in DMEM (Gibco, Grand Island, NJ, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco). The cells were cultured in the incubator with 5% CO_2 at 37°C. VSMCs were pretreated with liraglutide (Lira, 100 nM) for 24 h before being co-incubated with high glucose (HG) (30 mM) for 48 h. Cells from passages 3 to 5 were used throughout this study.

Detection of ROS

The ROS levels in the VSMCs were measured using a commercial ROS assay kit (Jiancheng Bioengineering Institute, Nanjing, China) by the manufacturer's instructions.

Cell Transfection

For overexpressing RCAN1, RCAN1 sequences were inserted into pcDNA3.1 vector (pc-RCAN1) (Invitrogen, Carlsbad, CA, USA). Empty pcDNA3.1 vector (pc-NC) was served as the negative control. Once the VSMCs reached 80% confluence, the cells were transfected using Lipofectamine 3000 Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). VSMCs were harvested for further experiments at 24 h post-transfection.

VSMCs Proliferation and Migration

VSMCs proliferation was measured using a Cell Counting Kit (CCK-8) assay (Jiancheng Bioengineering Institute) according to the manufacturer's instructions. Cellular migration was assessed using a wound-healing assay. VSMCs were seeded into six-well plates. When the cells reached 80% confluence, a scratch was made using a 200 mL pipette tip and washed with PBS to remove the debris. Cellular migration was assessed by light microscopy at 0 h and 24 h.

Western Blotting

Protein samples were obtained from cells or femoral tissues by homogenizing in T-PER reagent (Thermo Fisher Scientific, Waltham, MA, USA). The extract was centrifuged at 12,000g for 20 min at 4°C and the clear supernatant was isolated and evaluated for protein content by the BCA protein quantitative kit (Boster Technology Co., LTD, Wuhan, China). Equal quantities of protein (40 μg per group) were loaded and resolved on an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (PVDF membrane, 0.45 μm, Millipore Co. Ltd. Billerica, MA; USA). The membranes were blocked with 5% skim milk in Tris-buffered saline Tween-20 (TBST) for 2 h and then incubated with diluted primary antibodies against RCAN1 (NBP1-46853, Novus Biologicals, Littleton, CO, USA), PCNA (ab29, Abcam, Cambridge, UK), Collagen I (ab138492, Abcam), MMP-9 (#3852, Cell Signaling Technology, Danvers, MA, USA) and β-actin (ab8226, Abcam) overnight at 4°C. The membranes were then washed three times with TBST, incubated for an additional 2 h at 25°C with a secondary antibody (Sigma-Aldrich, St. Louis, MO, USA), and finally washed with TBST. The bands were exposed by the enhanced chemiluminescence method.

RT-qPCR

TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from cells. The RNA concentration was determined by NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). Extracted RNA was used as a cDNA synthesis template *via* the SuperScript II first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). cDNA was reversely transcribed *via* SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and Real-Time PCR System (Bio-Rad, Hercules, CA, USA) was conducted for quantitative real-time PCR. The PCR conditions for RCAN1 and GAPDH were as follows: denaturing, 90°C for 30 s; reverse transcription, 61°C for 20 min; preheating, 95°C for 1 min, then 45 cycles of 95°C for 15 s, 60°C for 30 s. The following primer pairs were designed: forward primer RCAN1 5'-GAAGATGCGACCCCAGTC-AT-3'; reverse primer 5'-CGCTGCGTGCAATTCAT-ACT-3'; and forward primer GAPDH 5'-CCT-GCACCACCAACTGCTTA-3'; reverse primer 5'-AGTGATGGCATGGACTGTGG-3'. The CT values of mRNA were analyzed by the $2^{-\Delta\Delta CT}$ method as the fold changes relative to β-actin.

Statistical Analysis

Our results were processed as mean ± SD (standard deviation). The statistical analyses were performed by GraphPad Prism software (La Jolla, CA, USA). For the comparisons between two or more sets, Student's *t*-test or one-way analysis of variance (ANOVA) was respectively applied to assess the statistical significance. $p < 0.05$ was considered to have statistical significance.

Results

Liraglutide Alleviated Intimal Hyperplasia and Dysfunction After Lower Limb Vascular Injury in Diabetes

To investigate the effect of Lira on the neointima after vascular injury in diabetes, a rabbit model of diabetes mellitus was established and treated with Lira. On the basis of successful modeling, the femoral arteries of rabbits were stained with HE, and the neointima/media ratios were calculated. As shown in Figure 1, the ratio of neointima/media of diabetic rabbits was significantly higher than that of the CTRL group ($p<0.001$), which suggested that Lira treatment significantly alleviated the hyperplasia of femoral artery intima caused by diabetes in rabbits.

Liraglutide

The levels of MDA and SOD in blood vessels of the lower limbs of rabbits were measured to

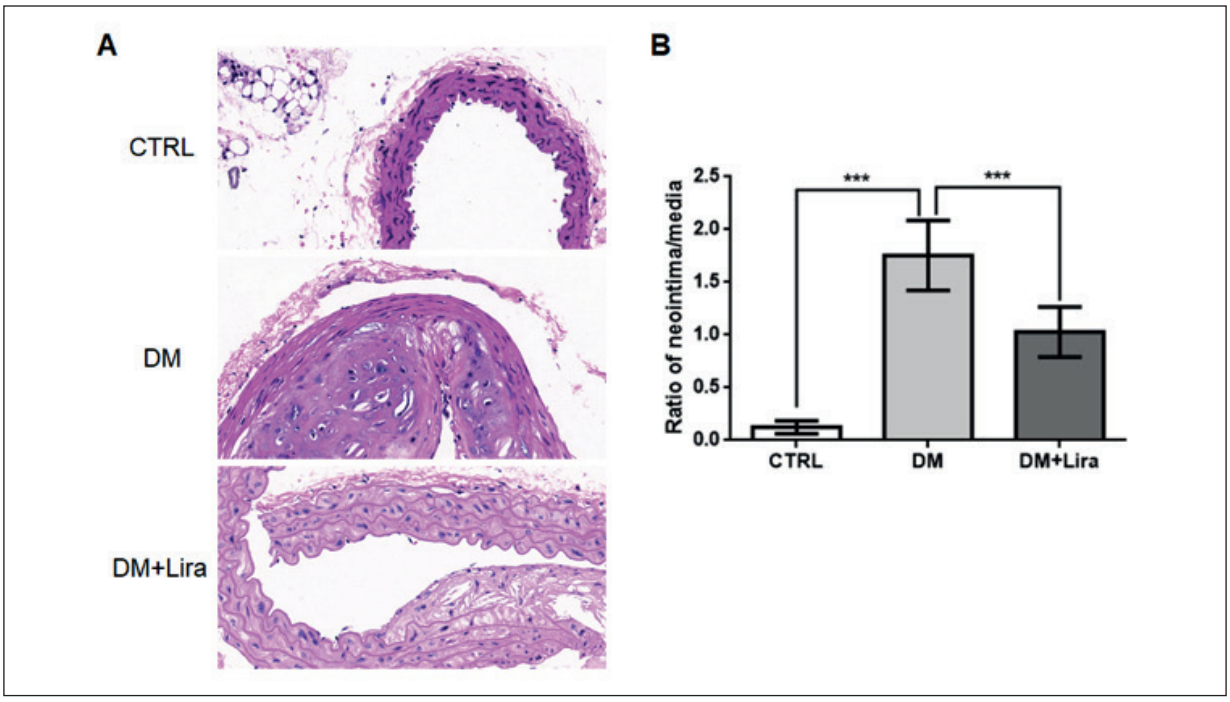

Figure 1. Lira inhibited neointimal proliferation after lower extremity vascular injury in rabbits. **A**, Blood vessel cross-section stained; **B**, Lira reduced the production of new intima after injury. ***p<0.001. Magnification: ×200.

determine the oxidative stress. Compared with normal rabbits, the MDA level of lower limb vascular tissue of diabetic rabbits was significantly increased (p<0.001), while the SOD level was significantly decreased (p<0.001, Figure 2A and 2B). Lira therapy appeared to significantly reverse MDA (p<0.05) and SOD (p<0.001) levels and alleviate oxidative stress caused by diabetes and vascular stenosis (p<0.001, Figure 2A and 2B).

In addition, to explore the therapeutic effect of Lira on vascular function, we detected the level of homeostasis model assessment of insulin resistance (HOMA-IR) in the vascular tissues of the lower limbs of rabbits. Diabetes mellitus and vascular stenosis significantly increased the level of HOMA-IR in lower limb vascular tissue of rabbits (p<0.001), which could be effectively alleviated by Lira treatment (p<0.001, Figure 2C). To further reveal the mechanism of action of Lira, we examined the expression level of RCAN1 protein in rabbit lower limb vascular tissue. Our results showed that compared with normal rabbits, significantly increased RCAN1 protein expression level in lower limb vascular tissues was observed in DM rabbits group (p<0.001), whereas Lira treatment was capable of significantly reducing RCAN1 expression level (p<0.01, Figure 2D).

Liraglutide Slowed the Proliferation and Migration of VSMCs Induced by High Glucose

In cell experiments, we treated high glucose (HG) induced VSMCs with Lira and detected proliferation and migration and other related properties of VSMCs. The ROS level of VSMCs were significantly increased by HG induction (p<0.001), but markedly reduced after Lira treatment (p<0.01, Figure 3A), suggesting that Lira inhibited HG-induced ROS overproduction. HG induced the proliferation of VSMCs and the expression level of proliferation-related factor PCNA increased (p<0.001, Figure 3B and 3E). However, Lira treatment significantly slowed down proliferation and inhibited the expression of PCNA in VSMCs (p<0.01, Figure 3B and 3E). HG induction also promoted the migration of VSMCs (p<0.01) and the expression of migration-related factor MMP-9 (p<0.001, Figure 3C, 3D and 3G), while Lira treatment could significantly inhibit the migration (p<0.01) and the expression of MMP-9 (p<0.001, Figure 3C, 3D and 3G). In

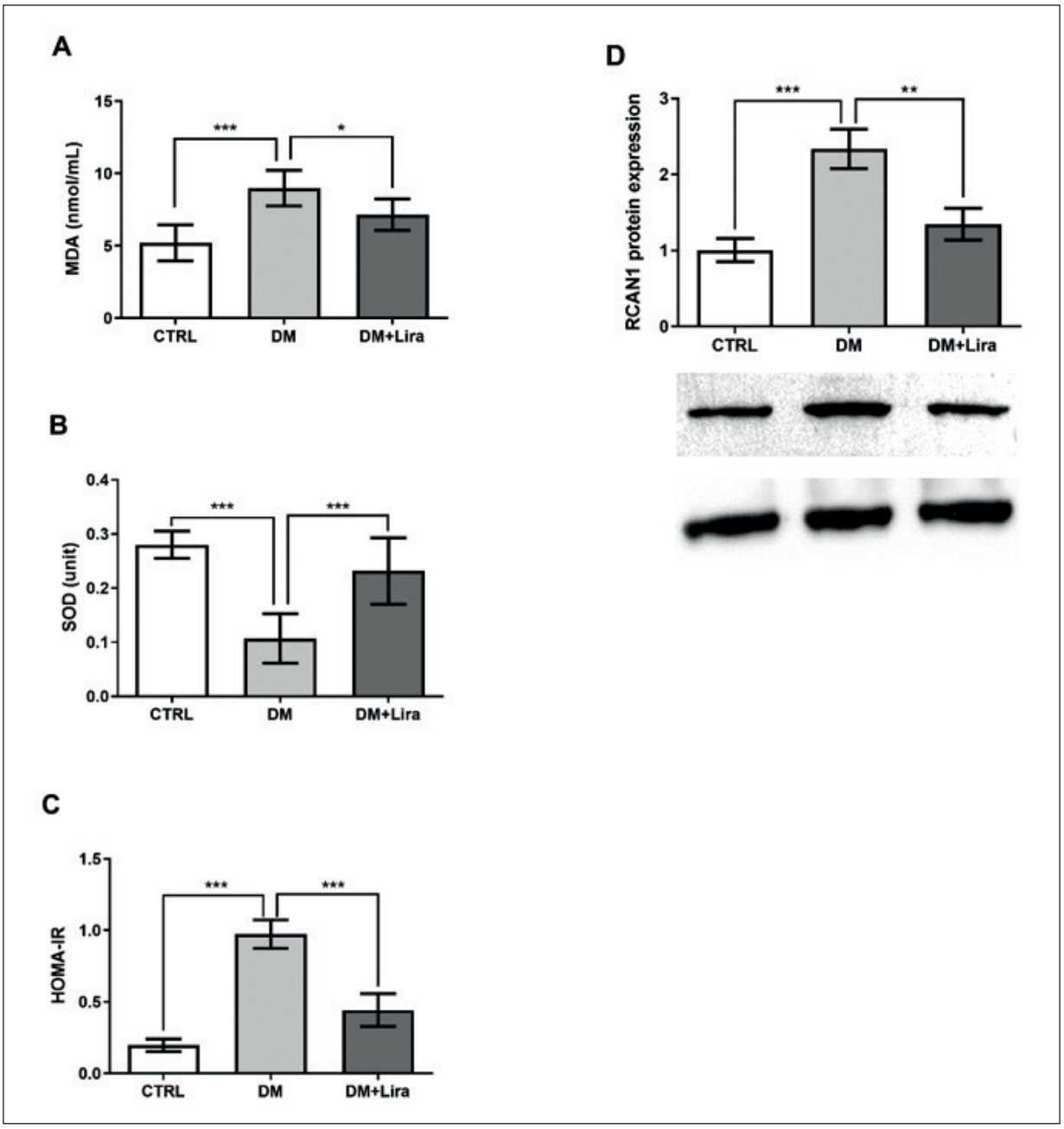

Figure 2. Lira alleviated lower limb vascular oxidative stress and vascular dysfunction in diabetic rabbits. **A**, Lira treatment significantly reduced the elevated MDA level of lower limb vascular tissue induced by diabetes in rabbits. **B**, Lira treatment reversed the decrease of SOD level in lower limb vascular tissue induced by diabetes in rabbits. **C**, Lira treatment significantly reduced the level of HOMA-IR in lower limb vascular tissue induced by diabetes in rabbits. **D**, LIRA treatment significantly inhibited the elevated expression of RCAN1 in lower limb vascular tissue induced by diabetes in rabbits. *p<0.05, ** p<0.01, ***p<0.001.

addition, to determine the extracellular mechanism of collagen deposition, we sought to detect the expression of collagen I in each group. Similarly, HG induction significantly increased the collagen I level of VSMCs (p<0.001), while contrary effects showed after Lira treatment (p<0.001, Figure 3F).

Liraglutide Inhibited HG-Induced Proliferation and Migration of VSMCs by Inhibiting RCAN1

Since RCAN1 expression was reduced by Lira treatment in animal experiments, we also detected RCAN1 expression levels in cells. The expression of RCAN1 mRNA in VSMCs were significantly

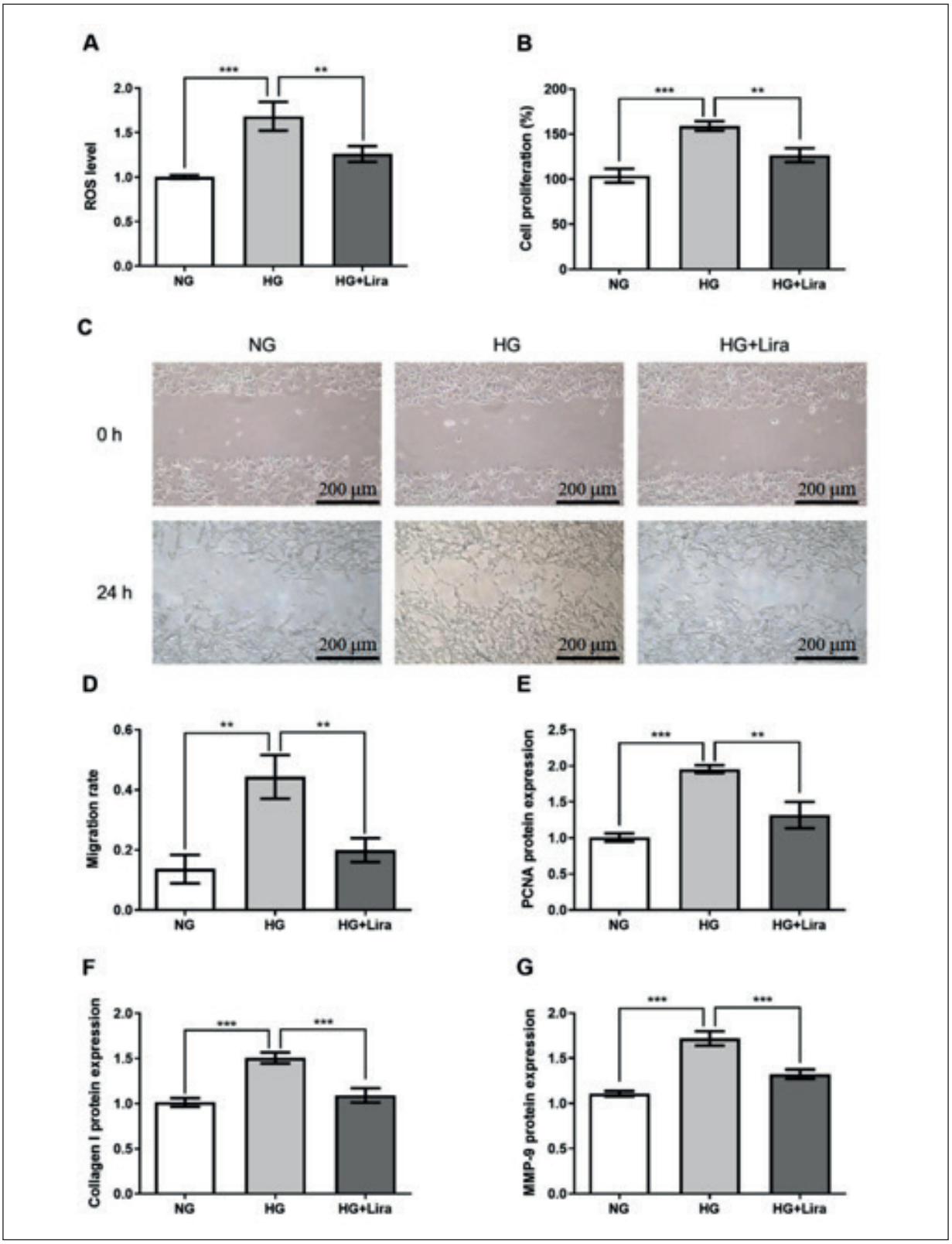

Figure 3. Lira slowed the proliferation and migration of VSMCs induced by HG. **A**, Lira inhibited the overproduction of ROS in HG-induced VSMCs. **B**, Lira inhibited the proliferation of VSMCs induced by HG. **C**, Representation of a cell wound-healing experiment (Magnification: ×200). **D**, Lira inhibited HG-induced VSMCs migration. **E**, Lira inhibited PCNA expression induced by HG. **F**, Lira inhibited HG-induced increase of collagen. **G**, Lira inhibited MMP-9 expression induced by HG. $^{**}p<0.01$, $^{***}p<0.001$.

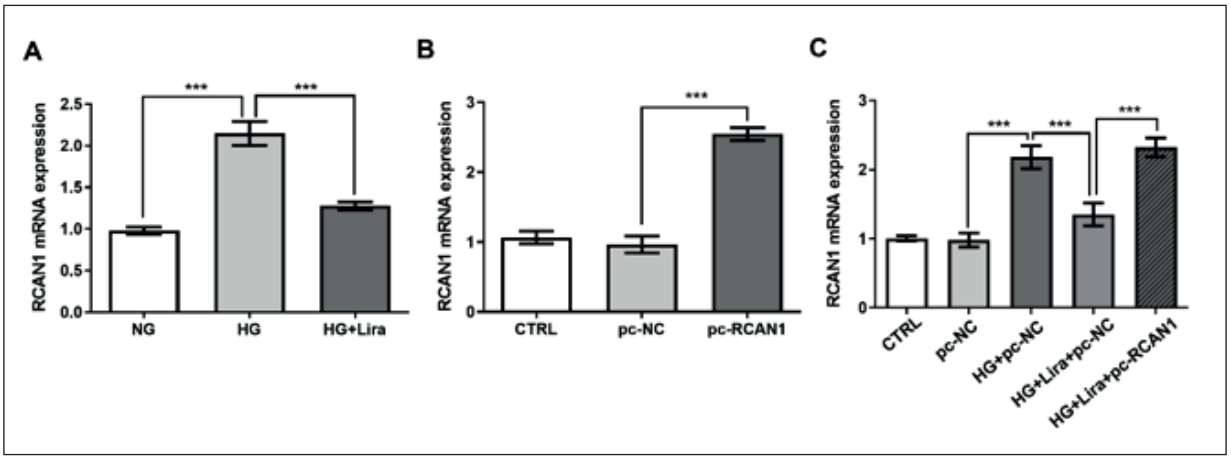

Figure 4. Modeling of the HG-induced VSMCs with RCAN1 overexpression. **A**, Lira inhibited the expression of RCAN1 in VSMCs induced by HG. **B**, The plasmid effectively increased the expression level of RCAN1 in VSMCs. **C**, RCAN1 mRNA expression level in each group. ***$p<0.001$.

increased by HG induction, while significantly decreased after Lira treatment ($p<0.001$, Figure 4A). To further reveal specific mechanism of the action of Lira, we constructed VSMCs transfected with RCAN1 overexpression plasmid. RCAN1 mRNA expression was detected by RT-qPCR, which confirmed that RCAN1 overexpression plasmid could effectively increase the expression level of RCAN1 in VSMCs ($p<0.001$, Figure 4B). After that, we constructed the HG induced VSMCs model with RCAN1 overexpression and detected the expression level of RCAN1 in cells to verify the success of modeling (Figure 4C).

The Proliferation and Migration and Other Related Properties of VSMCs in Different Groups

Lira treatment could reduce the accumulation of ROS induced by HG ($p<0.01$), and overexpression of RCAN1 in VSMCs could reverse the inhibition effect of Lira on ROS accumulation ($p<0.01$, Figure 5A). Lira significantly inhibited the proliferation of VSMCs and PCNA expression induced by HG ($p<0.001$), and overexpression of RCAN1 could eliminate the inhibitory effect of Lira ($p<0.01$ and $p<0.001$, Figure 5B and 5E). Lira significantly inhibited VSMCs migration and MMP-9 expression induced by HG ($p<0.01$), and overexpression of RCAN1 could also eliminate the inhibitory effect of Lira ($p<0.05$, Figure 5C, 5D and 5G). Lira treatment could reduce the increase of collagen I level induced by HG ($p<0.05$), and the overexpression of RCAN1 in VSMCs could counteract the inhibitory effect of Lira on collagen I accumulation ($p<0.05$, Figure 5F). Based on the above results, we believe that Lira is able to inhibit the proliferation and migration of VSMCs induced by high glucose *via* inhibiting the expression of RCAN1.

Discussion

Peripheral artery disease is one of the most common cardiovascular complications caused by diabetes. As a novel hypoglycemic drug, liraglutide can promote the efficiency of myocardial glucose uptake, and has advantages of cardiovascular protection, hypoglycemic and anti-inflammatory effects[6]. It has been reported that liraglutide has a therapeutic effect on peripheral vascular lesions caused by diabetes[11,12]. Despite that vascular interventional therapy is widely used in the treatment of peripheral vascular diseases of diabetes mellitus, the hyperplasia of intima has been the bottleneck after treatment. Liraglutide has a role in the regulation of glucose changes in diabetic pigs in a GLP-1 receptor-dependent manner and reduces hyperplasia after stent implantation[13]. The results of our study demonstrated that liraglutide showed an advantage in alleviating intima formation as well as restenosis of lower extremity vessels after lower extremity vascular injury in diabetic rabbits.

Oxidative stress is a key mechanism of diabetes-induced organ damage, which activates a series of inflammatory pathways and ultimately leads to intimal hyperplasia. A series of clinical

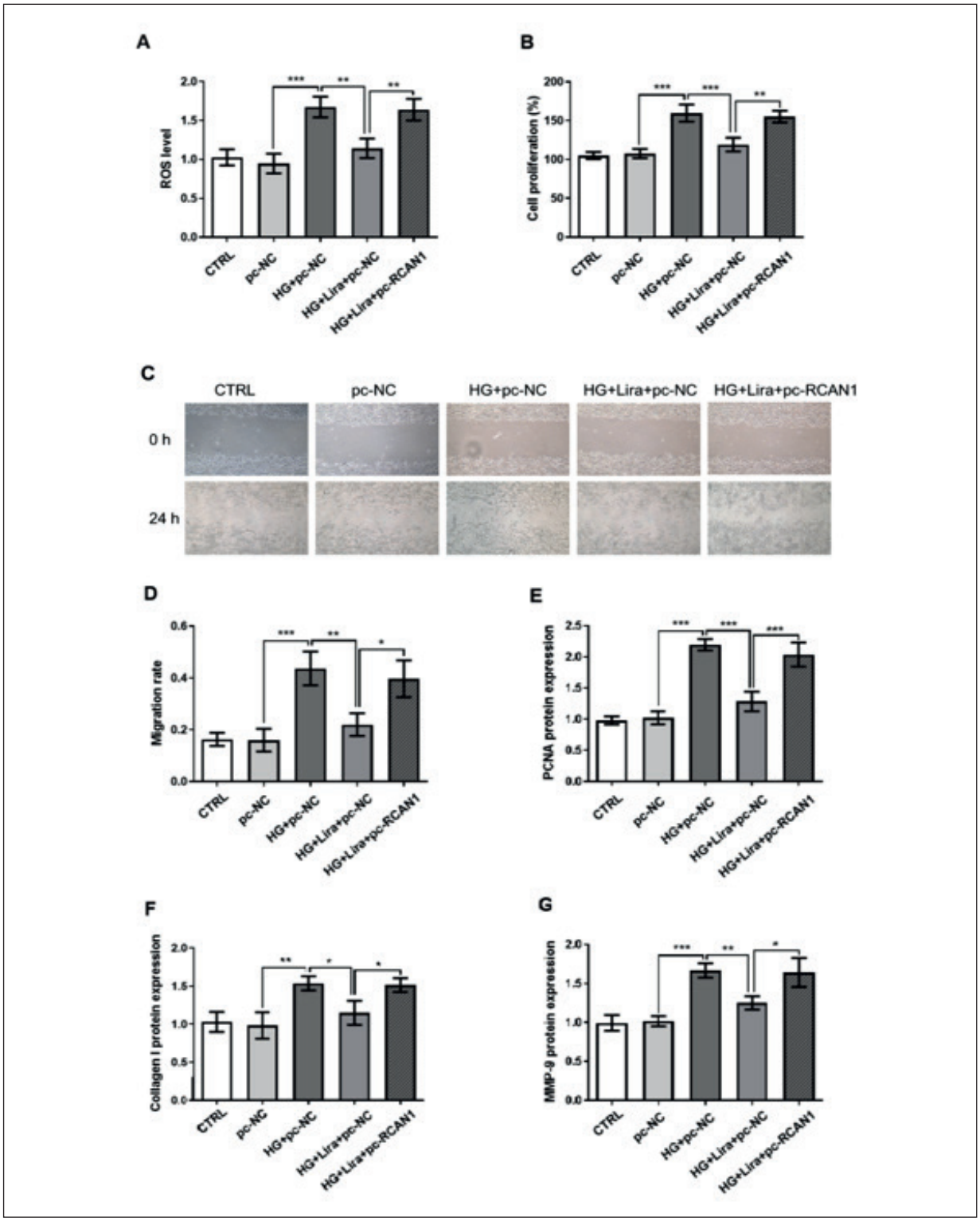

Figure 5. Lira inhibited the proliferation and migration of VSMCs induced by high glucose by inhibiting the expression of RCAN1. ***A,*** Overexpression of RCAN1 reversed the inhibition of ROS in HG-induced VSMCs by liraglutide. ***B,*** Overexpression of RCAN1 reversed the inhibitory effect of liraglutide on HG-induced VSMCs proliferation. ***C,*** Representation of a cell wound-healing experiment (Magnification: ×200). ***D,*** Overexpression of RCAN1 reversed the inhibitory effect of liraglutide on HG-induced VSMCs migration. ***E,*** Overexpression of RCAN1 reversed the inhibitory effect of liraglutide on HG-induced PCNA expression. ***F,*** Overexpression of RCAN1 reversed the inhibitory effect of liraglutide on HG-induced increase of collagen. ***G,*** Overexpression of RCAN1 reversed the inhibitory effect of liraglutide on HG-induced MMP-9 expression. *p<0.05, ** p<0.01, ***p<0.001.

and experimental studies confirmed the beneficial effects of GLP-1 receptor agonists on diabetes, and their independent glucose-lowering abilities, which are mediated by natriuresis, anti-inflammatory and anti-oxidative stress properties [14]. In our study, it was found that liraglutide treatment could significantly improve the vascular oxidative stress induced by diabetic lower extremity vascular stenosis in rabbits, which was manifested by decreasing MDA level and increasing SOD level. In addition, the HOMA-IR model revealed that liraglutide treatment significantly improved insulin hypersecretion after the induction of high glucose, presenting a state of continuous insulin resistance. Previous studies[17,18] have also shown that GLP-1 agonists contribute to sustained insulin resistance, which is consistent with our results.

Vascular remodeling is an important process in the development of peripheral artery disease, which are highly associated with pathologic changes in VSMCs, including proliferation, migration, and apoptosis[15,16]. Previous studies have suggested that HG concentrations promote these altered cellular behaviors in VSMCs. The main features of neointimal hyperplasia after vascular injury involves VSMC proliferation, migration, phenotypic transformation and collagen deposition of extracellular matrix. Our study found that liraglutide was able to evidently inhibit the proliferation and migration of VSMCs and the accumulation of extracellular matrix collagen induced by high glucose.

In this study, we were surprised to find that in both animals and cells, liraglutide treatment significantly reduced the expression level of RCAN1 in tissues or cells. By constructing RCAN1 overexpressing plasmid to transfection VSMCs, we found that liraglutide affected the proliferation and migration of VSMCs by regulating RCAN1, probably being one of the mechanisms of inhibiting intimal hyperplasia. It was observed that RCAN1 RNA expression was suppressed in glomeruli in human diabetic nephropathy and mouse models of HIV-associated nephropathy and diabetic nephropathy[19]. Epigenetic suppression of RCAN1 aggravated podocyte injury in the setting of HIV infection and diabetic nephropathy[19]. The association of RCAN1 with mitochondrial dysfunction in beta cells in Diabetes has been demonstrated[20]. The overexpression of the human RCAN1 in mice under the regulation of its endogenous promoter not only caused diabetes and age-associated hyperglycemia, but also led to reduced glucose tolerance and β-cell insulin secretion, hypoinsulinemia, loss of β-cells, aberrant mitochondrial reactive oxygen species production, and the down-regulation of key β-cell genes[21]. At the same time, one study reported that treatment of endothelial cells with GLP-1 significantly attenuated oxidative stress-induced endothelial dysfunction and autophagy, suggesting GLP-1 produces a protective effect on endothelial cells from oxidant injury by preventing endothelial dysfunction and autophagy[22]. It has also been reported by earlier study that liraglutide protects cardiomyocytes from mitochondrial dysfunction[23]. GLP-1 has beneficial effects for glucose homeostasis by stimulating insulin secretion from pancreatic beta-cells, delaying gastric emptying, decreasing plasma glucagon and food intake, and stimulating glucose disposal[24].

There are still several limitations in this study. For example, the downstream molecular mechanism of RCAN1 was not explored *in vitro*.

Conclusions

Liraglutide elicited advantages in alleviating the excessive formation of new intima and the restenosis of lower limb vessels after lower limb vascular injury in diabetic rabbits. *In vitro* results also demonstrated that liraglutide inhibited the proliferation and migration of VSMCs by inhibiting the expression of RCAN1 under high glucose treatment, suggesting that liraglutide may play a therapeutic role in the treatment of early endothelial injury and restenosis after intervention in diabetic lower extremity vascular disease. These findings offer a new insight to further explore the underlying mechanism by which Lira regulates VSMC cell migration in diabetic patients. Furthermore, diabetic lower extremity macrovascular lesions are mainly caused by atherosclerosis rather than neointimal hyperplasia. Intimal hyperplasia remains an important clinical problem after endovascular/open revascularization in response to pressure on the blood vessels, even in non-diabetic patients. Furthermore, the condition can be exacerbated among patients with diabetes or AIDS, making the maintenance of primary patency more challenging.

Conflict of Interest

The authors declare there is no conflict of interest.

Ethics Approval

The study protocol was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University.

Acknowledgments

None.

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