ORIGINAL RESEARCH

Liraglutide Attenuates Restenosis After Vascular Injury in Rabbits With Diabetes Via the TGF-β/Smad3 Signaling Pathway

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ABSTRACT

Background • Lower limb ischemia due to arterial stenosis is a major complication in patients with diabetes mellitus (DM). Liraglutide is a long-acting analogue of a glucagon-like peptide 1 (GLP-1) receptor agonist used for lowering blood glucose in patients with DM, and is believed to possess cardiovascular protective effects. The aim of this study was to investigate whether liraglutide has a protective effect on blood vessels and alleviates vascular intimal hyperplasia in streptozotocin (STZ)-induced rabbits with DM and its molecular mechanism.

Methods • Rabbits with DM were induced by STZ, and a lower limb ischemia model was established. The animals were divided into a control group, DM-injury group and liraglutide treatment group. Pathological staining was used to observe the intimal growth, analyze the oxidation levels of malondialdehyde (MDA), superoxide dismutase (SOD) and plasma glutathione peroxidase (GSH-Px), and analyze the changes in expression of marker proteins and signaling pathway proteins by Western blotting. A hyperglycemia (HG)-injured vascular smooth muscle cells (VSMCs) model was established to analyze reactive oxygen species (ROS) levels, Cell-Counting Kit-8 (CCK-8) was used to analyze cell proliferation, scratch assay and Transwell Migration Assay to analyze cell migration, flow cytometry to analyze apoptosis and Western blotting was used to analyze changes in the expression of marker and signaling pathway proteins.

Results • The results of pathological staining showed that intimal hyperplasia was severe after diabetes-induced lower limb ischemia in rabbits at 4 weeks, and liraglutide treatment reduced symptoms. Liraglutide treatment significantly decreased MDA content, increased SOD, GSH-Px content, and augmented total antioxidant capacity levels in tissues. The results of Western blotting analysis showed that E-cadherin, mitochondrial membrane potential 9 (MMP-9), proliferating cell nuclear antigen (PCNA), and type I collagen protein expression levels were significantly decreased after liraglutide treatment compared with the DM injury group. The results indicated that liraglutide inhibited epithelial-mesenchymal transition (EMT) progression, vascular cell proliferation and migration and collagen production. Liraglutide inhibits transforming growth factor beta 1 (TGF-β1)/Smad3 signaling pathway protein expression. *In vitro* assays have shown that liraglutide reduces cellular ROS levels, inhibits cell proliferation and migration and promotes apoptosis. Liraglutide down-regulated the expression of E-cadherin, MMP-9, PCNA, type I collagen protein as well as the TGF-β1/Smad3 signaling pathway, but this effect could be reversed by tumor necrosis factor alpha (TNF-α).

Conclusion • Liraglutide can significantly improve tissue antioxidant capacity, reduce vascular cell proliferation and migration via the TGF-β1/Smad3 signaling pathway, inhibit the EMT and collagen production processes, and alleviate hyperglycemia(HG)-induced lower limb ischemia and intimal hyperplasia. (*Altern Ther Health Med.* 2022;28(6):22-28)

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INTRODUCTION

Lower limb ischemia due to arterial stenosis is a major complication in patients with diabetes mellitus (DM), and despite current advances in clinical trials, the amputation rate remains high.[1] In addition, the risk for restenosis in patients with DM after percutaneous transluminal coronary angioplasty is higher than in patients without DM. In addition, DM is one of the most important vascular risk factors for cerebral microangiopathic infarction.[2]

Restenosis after vascular reconstruction, mainly arising from neointimal hyperplasia, is associated with abnormal proliferation and migration of vascular smooth muscle cells (VSMCs). Therefore, inhibition of VSMCs proliferation and migration is a key therapeutic strategy for angioplasty-associated restenosis. Hyperglycemia (HG) in the blood of patients with DM often results in increased production of reactive oxygen species (ROS), which stimulate the proliferation and migration of VSMCs, thus leading to VSMC accumulation in the vascular intima.[3] We predicted that the local redox imbalance of the vascular wall is effectively improved in patients with DM, which may help delay or even prevent restenosis.

Glucagon-like peptide 1 (GLP-1) plays an important role in the pathogenesis of diabetic atherosclerosis. It also prevents vascular remodeling and protects endothelial cells from oxidative stress by improving the intimal inflammatory response. Liraglutide, one of the GLP-1 receptor agonists, has been shown to reduce carotid intima media thickness in patients with type 2 diabetes (T2D).[4] *In vitro* experiments showed that liraglutide treatment decreased ERK1/2 and Akt phosphorylation, inhibited cell migration and proliferation and increased apoptosis in HG-induced VSMCs by activating GLP-1 receptors, and that liraglutide itself did not induce physiological changes in VSMCs and could only inhibit HG-induced effects.[5] Transforming growth factor β1(TGF-β1) is an important initiator of intracellular signaling pathways, and has been reported to induce the expression of extracellular matrix (ECM) proteins in cardiac fibroblasts by activating Smads-dependent signaling in mice with DM, which causes pathological fibrosis.[6] However, the mechanism of TGF-β/Smad3 signaling in diabetic vascular restenosis has not been reported.

The aim of this study was to investigate the role of liraglutide in improving lower limb vascular disease in patients with DM and identify the associated molecular mechanisms.

MATERIALS AND METHODS

Animal Model Establishment

The lower limb ischemia model of rabbits with T2D was established: rabbits with a body weight of 2.5 to 3.0 kg were fed a high-fat diet (25%) for 2 months and intraperitoneal injection of 0.05 mM streptozotocin 60 mg/kg. Rabbits with fasting blood glucose higher than 16.7 mM were selected to establish the lower limb ischemia model. Rabbits were randomly divided into the normal group, the ischemia model group and the liraglutide ischemia group by the random number method, with 6 rabbits in each group. Intraperitoneal injection of 3% pentobarbital sodium (1 ml /kg) was used for anesthesia. The left femoral artery was carefully exposed at least 1 cm, and 2.5 cm was cut from the middle of the right groin to the medial side of the knee joint and separated from the surrounding tissue. Then the femoral artery was ligated to establish the ischemia model. Incisions were sutured and gentamicin was administered to prevent infection. The normal group and untreated diabetic ischemic injury group were given the same amount of normal saline. The calculation formula of sample size was $n = Z^2 \times [P \times (1\text{-}P)]/E^2$. Where n = sample size; Z = statistic, Z =1.96 when the CI is 95%; z = 164 when the CI is 90%; E = error value; *P* = probability value.

The minimum sample size for this quantitative study was 18 samples. Liraglutide, clinically used for T2D treatment, was given to the rabbits with lower extremity ischemia at a dose of 0.6 mg per day by subcutaneous injection at the subcutaneous site of the abdomen for 1 week.

Ethics Statement

All research involving animals complied with the protocols approved by The Fourth Hospital of Hebei Medical University and all the rabbits were purchased from the animal center.

Histopathology Stain

Skeletal muscle tissues from the left lower limb were routinely fixed, dehydrated, embedded and paraffin sections were made. Sections were mounted on slides and stained with hematoxylin and eosin (H&E) stain. H&E was carried out according to the protocol supplied by the manufacturer (Abcam, Cambridge, Massachusetts, USA). The cross-sectional area of media and intima was measured by Image J software (NIH, Littleton, Colorado, USA) and the intima/media ratios were calculated.

Verhoeff-van Giesonsstain(EVG) is used for evaluation of arterial intimal fibrosis. EVG was conducted according to the staining kit protocol (Abbexa, Cambridge, UK). Proliferating cell nuclear antigen (PCNA)-positive(+) cells were visualized by a TRITC epifluorescence filter and identified by the presence of a bright green nuclear stain. PCNA was conducted according to the staining kit protocol from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Nuclei were identified by 4',6-diamidino-2-phenylindole (DAPI) stain and the procedure was carried out according to the instruction manual of the DAPI staining kit (Abbexa, UK).

Detection of MDA, SOD and GSH-Px in Arterial Tissues

The concentration of malondialdehyde (MDA) was determined to assess the level of lipid peroxidation. The level of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity was measured to evaluate the antioxidant levels. Commercial assay kits used were purchased from Nanjing Jiancheng Bioengineering Institute and used in accordance with the manufacturer's instructions.

VSMCs culture and treatment

Human aortic smooth muscle cells (VSMCs) were purchased from Cell Application at passage 4, cultured in DMEM (Gibco, Grand Island, NY, United States) supplemented with 10% fetal bovine serum (Gibco, United States), 100 U/ml penicillin (Gibco, United States) and 100 μg/ml streptomycin (Gibco, United States). The cells

were cultured in an incubator with 5% CO_2 at 37°C. VSMCs were pretreated with liraglutide (Lira; 100 nM) for 24 hours and/or recombinant TGF-β1 (10 ng/mL) before being co-incubated with HG (30 mM) for 48 hours. Cells from passages 3 to 5 were used throughout this study.

Detection of ROS

VSMCs cells in 12 petri dishes were taken for ROS detection. The ROS levels in the VSMCs were measured using a commercial ROS assay kit (Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's instructions.

Cell Counting Kit-8 Assay

VSMCs cells in 12 petri dishes were selected for Cell Counting Kit-8 (CCK-8) detection. VSMC proliferation was measured using a CCK-8 assay (Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

Wound Healing Analysis

VSMCs were seeded into 6-well plates. When the cells reached 80% confluence, a scratch was made using a 200 mL-pipette tip and washed with phosphate buffered saline (PBS) to remove the debris. Cellular migration was assessed by light microscopy at 0 and 24 hours.

Transwell Assay

VSMC migration was evaluated using a Transwell chamber assay (8-mm pore size; Millipore, Billerica, Massachusetts, USA). VSMCs were seeded into the upper chamber. After treatment and 24-hour incubation, the cells that migrated to the lower side were fixed with methanol, stained with 0.1% crystal violet and enumerated using light microscopy.

Flow Cytometry

Cell apoptosis was determined using the annexin V-FITC apoptosis assay kit (Nanjing Jiancheng) according to the instructions. VSMCs (1×10^5 cells/well) was trypsinized and centrifuged at 1000 g/min for 5 min. The cells were centrifuged and resuspended twice in phosphate-buffered saline (PBS), followed by a third centrifugation, and then the cells were resuspended in 100 μL of binding buffer (5×10^5 cells/100 μL). After incubation with 5 μL of annexin V-FITC and 5 μL of propidium iodide (PI) staining solution for 10 min in the dark, the cells were determined by FACS Canto II flow cytometry (BD Biosciences, San Jose, California, USA) within 1 hour. FlowJo™ 7.6 software was used to analyze the data and the percentage of both early and late apoptosis cells to total cells was calculated as the ratio of apoptotic cells.

Western Blotting

Serum vessel tissue samples were collected 1 week after injury. A protein sample was obtained from cells or carotid tissues by homogenizing in T-PER reagent (Thermo Fisher Scientific, Massachusetts, USA). The extract was centrifuged at 12 000 g for 20 minutes at 4°C and the clear supernatant was isolated and evaluated for protein content by the BCA Protein Quantitative Kit (BU Technology CO, LTD). Equal quantities of protein (40 μg per group) were loaded and resolved on an SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (PVDF membrane, 0.45 μm; Millipore Co. Ltd.). The membranes were blocked with 5% skim milk in Tris-buffered saline Tween-20 (TBST) for 2 hours and then incubated with diluted primary antibodies against PCNA (dilution ratio 1:400; ab29, abcam), type I Collagen (dilution ratio 1:400; ab138492), E-cadherin (dilution ratio 1:400; #3195, Cell Signaling Technology), MMP-9 (dilution ratio 1:400; #3852, Cell Signaling Technology, Danvers, Massachusetts, USA), TGF-β1 (dilution ratio 1:400; ab179695), p-Smad3 (dilution ratio 1:400; #9520, Cell Signaling Technology), Smad3 (dilution ratio 1:400; #9523, Cell Signaling Technology) and β-actin (dilution ratio 1:400; ab8226, Abcam, Cambridge, UK) overnight at 4°C. The membranes were then washed 3 times with TBST, incubated for an additional 2 hours at 25°C with a secondary antibody (Sigma-Aldrich, St Louis, Missouri, USA), and finally washed with TBST. The dilution ratio of the second antibody was 1:400. The bands were exposed by the enhanced chemiluminescence method.

Table. Blood Glucose Levels in the Three Groups of Rabbits ($x \pm s$, n = 6, mM)

Groups	Blood Glucose Levels
Normal	5.23±0.52
Ischemia model	12.96±2.10
Liraglutide ischemia	6.11±0.97
t	-12.277
P value	<.001

Statistical analysis

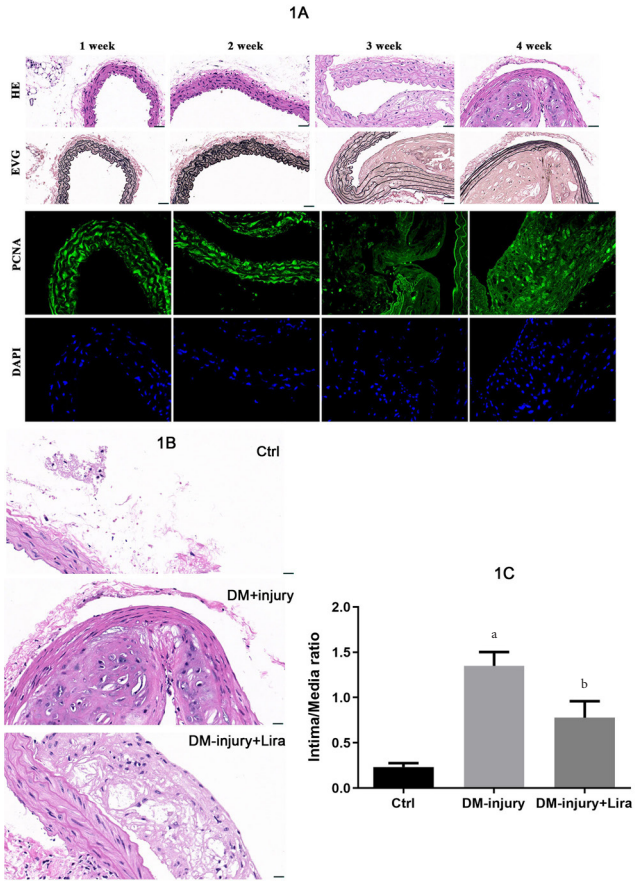
Results were processed as mean ± SD (standard deviation). The statistical analyses were performed by GraphPad Prism software (San Diego, California, USA). First, the data were tested for normality, and all the tested data were in line with normal distribution. For the comparisons between ≥2 sets, student's *t* test or one-way analysis of variance (ANOVA) was applied, respectively, to assess the statistical significance. LSD-T test was used for pairwise comparison between groups. $P < .05$ was considered statistically significant.

RESULTS

Liraglutide Inhibits Hyperplasia Neointima After Lower Limb Vascular Injury

Histological analysis found that hyperplasia neo-intima caused by DM injured vascular significantly at 4 weeks. The results suggested that DM-induced angio-obliterative lesions were formed (Figure 1A). The intima/media ratio (IMR) was measured to evaluate the effect of liraglutide on neo-intima hyperplasia induced by DM. The results showed that the IMR declined sharply after liraglutide therapy compared with DM (Figure 1B, 1C), which means liraglutide helped reduce the occurrence of hyperplasia neo-intima caused by DM.

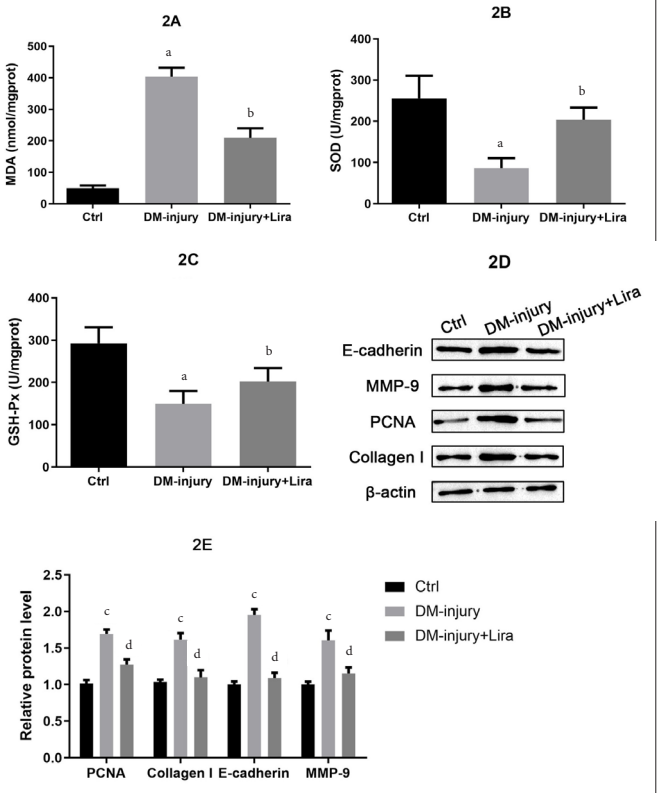
Figure 1. Liraglutide inhibits neointima hyperplasia after lower limb vascular injury. (1A) H&E, EVG, PCNA and DAPI staining of vascular DM injury at 1, 2, 3 and 4 weeks (400-fold magnification). (1B) Representative images of H&E staining of vascular injury in the control, DM-injury and DM-injury with liraglutide therapy groups at 4 weeks (400-fold magnification). (1C) Statistical analysis of the intima/media ratio (IMR)



[a]$P < .001$, Control group vs DM-injury
[b]$P < .001$, DM-injury vs DM-injury+Lira

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole stain; DM, diabetes mellitus; EVG, Verhoeff-van Giesonsstain stain; H&E, hematoxylin and eosin stain; Lira, liraglutide; PCNA, proliferating cell nuclear antigen.

Figure 2. Liraglutide attenuates oxidative stress and improves vascular injury caused by DM. (2A) Effects of DM and liraglutide treatment on MDA activity. (2B) Effects of DM and liraglutide treatment on SOD activity. (2C) Effects of DM and liraglutide treatment on GSH-Px activity. (2D) Statistical analysis for the protein expression of PCNA, type I collagen, E-cadherin and MMP-9. (2E) Protein expression was analyzed by Western blotting for PCNA, type I collagen, E-cadherin and MMP-9



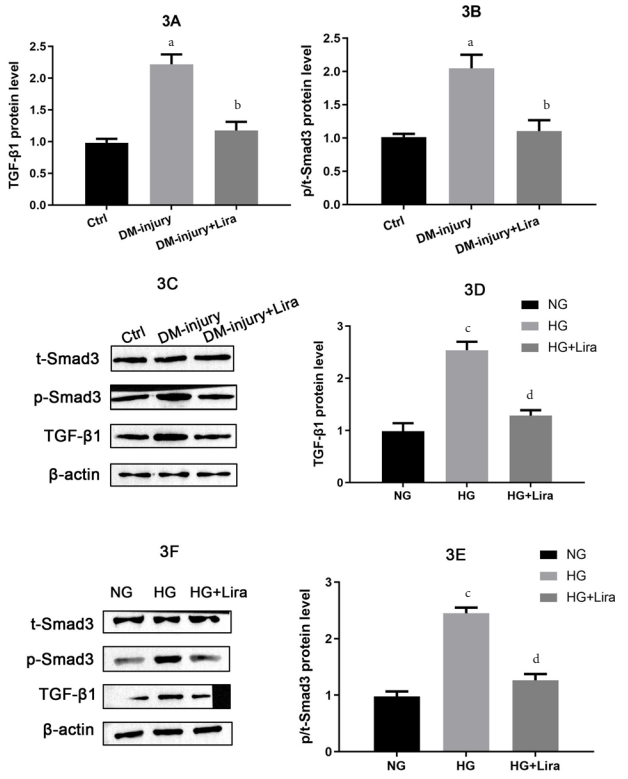
[a]$P < .001$, Control vs DM-injury
[b]$P < .05$, DM-injury vs DM-injury+Lira
[c]$P < .01$, DM-injury vs DM-injury+Lira
[d]$P < .001$, DM-injury vs DM-injury+Lira

Abbreviations: DM, diabetes mellitus; GSH-Px, plasma glutathione peroxidase; Hg, high glucose; Lira, liraglutide; MDA, malondialdehyde; MMP-9, mitochondrial membrane potential; NG, normal glucose; PCNA, proliferating cell nuclear antigen; SOD, superoxide dismutase.

Liraglutide Attenuates Oxidative Stress and Improves Vascular Injury Caused by DM

The results showed that DM caused significant effects on increasing MDA and decreasing SOD and GSH-Px compared with the control group. However, the decreased SOD and GSH-Px can be improved with liraglutide and in the meantime reduce the MDA content. These results suggested liraglutide can play a role in protecting antioxidant enzyme activity and inhibit MDA production (Figure 2A-2C). In order to analyze the effect of persistent hyperglycemia on vascular tissue, Western blotting was used to determine the expression of epithelial to mesenchymal transition (EMT) biomarkers E-cadherin and migration-related factor MMP-9, proliferation markers of cell nuclear antigen (PCNA) and collagen production related protein Collagen I. The results showed that DM induced these proteins to be upregulated, which could be reversed by liraglutide treatment (Figure 2D, 2E). E-cadherin and MMP-9, PCNA and type I collagen were significantly reduced with liraglutide treatment, which means that liraglutide has an effect on DM-induced vascular injury.

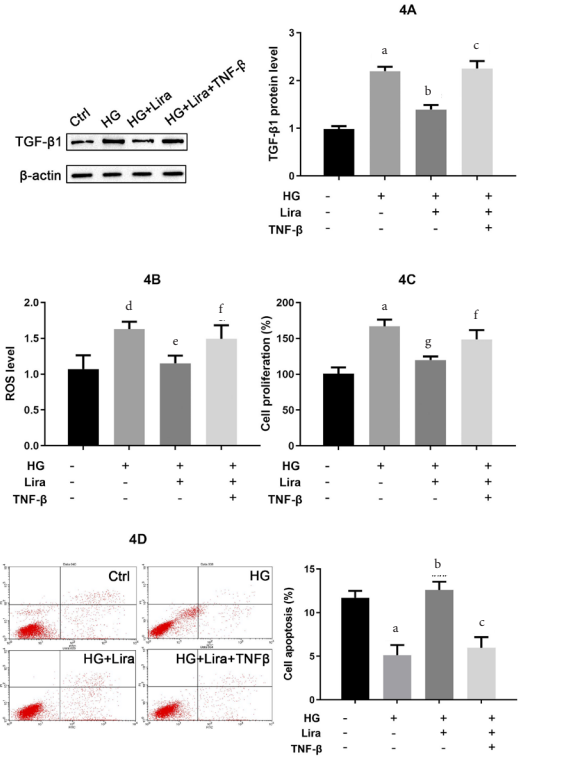
Figure 3. Liraglutide ameliorates DM-induced vascular injury *in vivo* and *in vitro*. (3A) Statistical analysis of the protein expression of TGF-β1 and (3B) p/t-Smad3 in different groups of rabbit vascular. (3C) Western blotting analysis of the expression of TGF-β1 and p/t-Smad3. (3D) Statistical analysis of the protein expression of TGF-β1 and (3E) p/t-Smad3 in different VSMC groups. (3F) Western blotting analysis of the expression of TGF-β1 and p/t-Smad3.



[a]*P* < .001, Control vs DM-injury
[b]*P* < .001, DM-injury vs DM-injury+Lira
[c]*P* < .001, NG vs HG
[d]*P* < .001, HG vs HG+Lira

Abbreviations: DM, diabetes mellitus; HG, hyperglycemia; Lira, liraglutide; NG, normal glucose; TGF-β1, transforming growth factor β1; VSMC, vascular smooth muscle cells.

Figure 4. TNF-β reversed the inhibitory effect of liraglutide on HG-induced VSMC proliferation and decreased ROS level. (4A) Expression level of TGF-β1 analyzed by Western blotting. (4B) ROS level in different VSMC groups. (4C) CCK-8 analysis of the ability of VSMC proliferation. (4D) Analysis of VSMC apoptosis by flow cytometry.



[a]*P* <.001, NG vs HG
[b]*P* <.001, HG vs HG+Lira
[c]*P* <.001, HG+Lira vs HG+Lira+TNF-β
[d]*P* <.01, NG vs HG
[e]*P* <.05, HG vs HG+Lira
[f]*P* <05, HG+Lira vs HG+Lira+TNF-β
[g]*P* <.01, HG vs HG+Lira

Abbreviations: CCK-8, Cell-Counting Kit-8; DM, diabetes mellitus; HG, hyperglycemia; Lira, liraglutide; NG, normal glucose; ROS, reactive oxygen species; TGF-β1, transforming growth factor β1; VSMC, vascular smooth muscle cells.

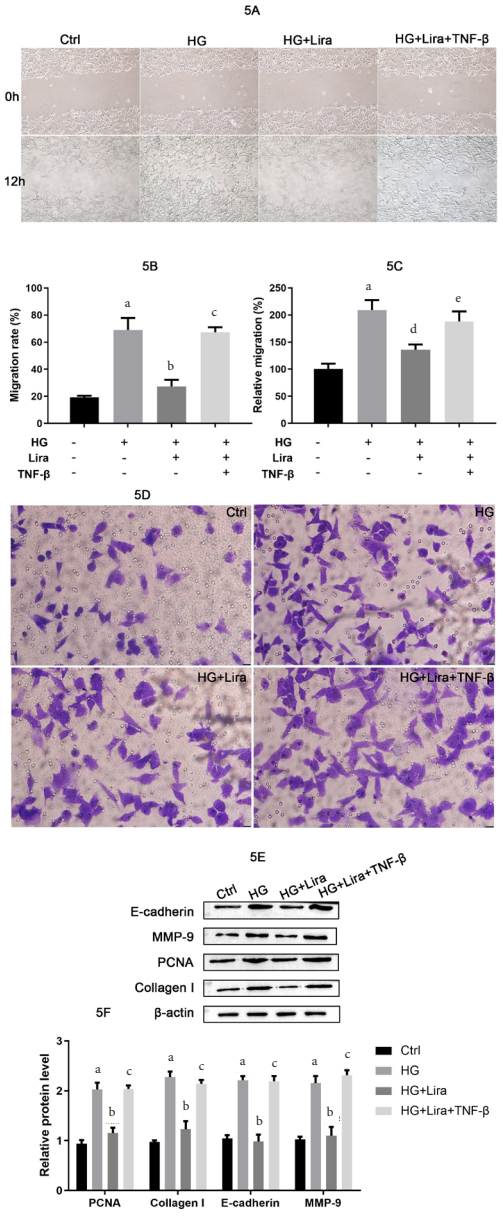
Liraglutide Ameliorates DM-induced Vascular Injury by Regulating TGF-β1/Smad3 Signaling

The expression levels of TGF-β1, p-Smad3 and total Smad3 were increased in vascular tissue from DM rabbits compared with controls, and treatment with liraglutide decreased TGF-β1, p-Smad3 and total Smad3 levels (Figure 3A-3C). Establishment of a vascular cell injury model induced by HG *in vitro* and the expression level of TGF-β1 and Smad3 were detected by Western blotting. The results showed that HG induced TGF-β1 and Smad3 upregulation, which were decreased after liraglutide treatment (Figure 3D-3F). Liraglutide may reduce vascular intimal hyperplasia by inhibiting the TGF-β1/ Smad3 pathway.

Tumor Necrosis Factor-β (TNF-β) Reversed the Inhibitory Effect of Liraglutide on HG-induced VSMC Proliferation and Decreased ROS

The results of Western blotting showed that TNF-β treatment reversed the inhibitory effect of liraglutide on TGF-β1 in HG-induced VSMCs (Figure 4A). The results of ROS assay suggested that ROS was increased in HG-induced VSMCs, which was decreased by liraglutide treatment, while TNF-β treatment reversed the inhibitory effect of liraglutide on ROS in cells (Figure 4B). Furthermore, the proliferation ability of VSMCs was determined by CCK-8 and it was found that HG promoted cell proliferation and liraglutide treatment inhibited the VSMC proliferation, while TNF-β treatment promoted the

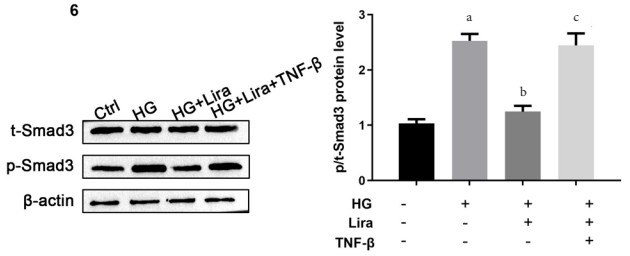
Figure 5. Liraglutide inhibits HG-induced migration of VSMCs by down-regulating TNF-β. (5A) Representative images of scratch assay. (5B) Statistical analysis of cell migration rate (%) and (5C) relative migration (%). (5D) Transwell migration assay of VSMCs. (5E-5F) Western blotting analysis of the protein expression of PCNA, type I collagen, E-cadherin and MMP-9.



[a]$P < .001$, NG vs HG
[b]$P < .001$; HG vs HG+Lira
[c]$P < .001$, HG+Lira vs HG+Lira+TNF-β
[d]$P < .01$, HG vs HG+Lira
[e]$P < .05$, HG+Lira vs HG+Lira+TNF-β

Abbreviations: DM, diabetes mellitus; HG, hyperglycemia; Lira, liraglutide; NG, normal glucose; PCNA, proliferating cell nuclear antigen; TNF-β, lymphotoxin-α; VSMC, vascular smooth muscle cells.

Figure 6. Western blotting analysis of the protein expression of t/p-Smad3



[a]$P < .001$, NG vs HG
[b]$P < .001$, HG vs HG+Lira
[c]$P < .001$, HG+Lira vs HG+Lira+TNF-β

Abbreviations: HG, hyperglycemia; Lira, liraglutide; NG, normal glucose; TNF-β, lymphotoxin-α.

VSMC proliferation (Figure 4C). TNF-β reversed the promotion effect of liraglutide on cell apoptosis (Figure 4D). These findings indicate that liraglutide improves HG-induced VSMC injury by downregulating TGF-β1, inhibiting ROS in cells and proliferation and promoting VSMC apoptosis.

Liraglutide Inhibits HG-induced Migration of VSMCs by Down-regulating TNF-β

Scratch test results showed that the migration ability of HG-induced VSMCs was enhanced, and liraglutide treatment inhibited VSMCs migration, while TNF-β promoted VSMCs migration ability (Figure 5A, 5B). To validate these findings, Transwell assay was performed and we had consistent results. Liraglutide treatment decreased the number of migrated VSMCs, induced by HG, but TNF-β exerted the opposite effect (Figure 5C, 5D). Then, Western blotting was used to analyze the expression of PCNA, type I collagen, E-cadherin and MMP-9, and we found that liraglutide treatment protects VSMCs from HG injury by downregulating the expression of PCNA, type I collagen,, E-cadherin and MMP-9 (Figure 5E, 5F). These data indicate that VSMC proliferation ability, migration ability, EMT progression and collagen production were enhanced by HG. TNF-β plays the opposite role.

Liraglutide Inhibits Smad3 Activation in HG-induced VSMCs and TNF-β Plays the Opposite Role

To further verify this result, t/p-Smad3 expression level was detected by Western blotting. The result was in accordance with the above results. In summary, liraglutide inhibits the TGF-β1/Smad3 pathway in HG-induced VSMCs. However, TNF-β upregulated the expression of TGF-β1/Smad3 and TNF-β activated the TGF-β1/Smad3 pathway.

DISCUSSION

In this study, we explored the efficacy of liraglutide in reducing the occurrence of hyperplasia neointima caused by DM and its underlying mechanisms. Our findings showed that liraglutide plays a role in protecting antioxidant enzyme activity and inhibiting MDA production. And liraglutide inhibits the

EMT process of vascular cells and vascular cell proliferation and migration, as well as collagen production in animals with DM. Our *in vitro* experiments showed that TNF-β reversed the inhibitory effect of liraglutide on HG-induced VSMC proliferation and decreased the ROS level. Furthermore, our study suggested that liraglutide inhibits the TGF-β1/Smad3 pathway in HG vascular cells, which protects vascular cells from HG injury.

Hyperglycemia and systemic inflammation caused by DM can lead to endothelial dysfunction.[7] Endothelial damage is established as a severe risk factor for diabetic vascular complications, and vascular protection in DM has been receiving much attention. Takahashi, et al found that combined treatment with linagliptin and empagliflozin attenuates neointima formation after vascular injury in mice with DM.[8] Liraglutide is a long-acting analogue of a GLP-1 receptor agonist, is used for lowering blood glucose in patients with DM, and is believed to possess cardiovascular protective effects.[9] Tsai, et al demonstrated that liraglutide inhibits vascular EMT and reduces neointima formation in mice with diabetes.[10] A previous study found that liraglutide can ameliorate endothelial endoplasmic reticulum stress in patients with DM.[11] Their results were in line with our study, which showed that liraglutide has an effect on inhibiting the EMT process, proliferation and migration of vascular cells in rabbits with DM.

Reports indicated that TGF-β1/Smad3 acts as a key mediator in the progression of DM,[12] and it is also reported that TGF-β1 was upregulated and promoted the EMT process.[13] Xie, et al proposed that lycopus extracts ameliorate podocytes injury by inhibiting the TGF-β1 signaling pathway of diabetic nephropathy in rats.[14] TGF-β1 is the main inducer and modulator of pathophysiological processes in diabetic nephropathy.[15] In our study, liraglutide inhibited the TGF-β1/Smad3 pathway in HG-induced VSMCs, consistent with other studies. Liraglutide was reported to suppress TGF-β1-induced phosphorylation of Smad3.[16] Recent studies revealed that TGF-β1/Smad3 is very important in promoting β-cell apoptosis and it a potential DM therapeutic target to restore β-cell mass loss.[17]

TNF-β as an inflammatory mediator has been reported to promote tumorigenesis and is a type of cytokine with a wide range of inflammatory and immunomodulatory activities. It is proposed that resveratrol down-regulates TNF-β-induced EMT in cancer stem cells.[18] Studies have shown that TNF-α activates epithelial and endothelial cells to produce interleukin-1 and promote the development of inflammation.[19] Studies have proposed that TNF is involved in the main proinflammatory factors of pancreatic β-cell destruction, while the interaction of interleukin-1 and interferon promotes TNF cytotoxicity.[20] Therefore, this study speculated that glucagon-like peptide 1(GLP-1) plays an important role in the pathogenesis of diabetic atherosclerosis, and GLP-1 plays a key role in this process, protecting endothelial cells from oxidative stress and down-regulating their apoptosis level.

CONCLUSION

In conclusion, our study found that TNF-β can reverse the effect of liraglutide on HG-induced VSMCs. This could possibly be due to the fact that TNF-β is an inflammatory cytokine with a wide range of functions. Liraglutide inhibits hyperglycemia-induced VSMC proliferation and migration and promotes apoptosis by inhibiting the TGF-β1/Smad3 pathway and thus alleviates excessive neointima formation after HG injury. TNF-β reversed the inhibitory effect of liraglutide on abnormal proliferation and migration of VSMCs.

CONFLICT OF INTEREST

None.

REFERENCES

1. Jiang X, Yuan Y, Ma Y, et al. Pain management in people with diabetes-related chronic limb-threatening ischemia. *J Diabetes Res*. 2021;2021:6699292. doi:10.1155/2021/6699292
2. Lee J-I, Gemerzki L, Weise M, et al. Retinal layers and visual conductivity changes in a case series of microangiopathic ischemic stroke patients. *BMC Neurol*. 2020;20(1):333-333. doi:10.1186/s12883-020-01894-y
3. Ariyanti AD, Zhang J, Marcelina O, et al. Salidroside-pretreated mesenchymal stem cells enhance diabetic wound-healing by promoting paracrine function and survival of mesenchymal stem cells under hyperglycemia. *Stem Cells Transl Med*. 2019;8(4):404-414. doi:10.1002/sctm.18-0143
4. Rizzo M, Chandalia M, Patti AM, et al. Liraglutide decreases carotid intima-media thickness in patients with type 2 diabetes: 8-month prospective pilot study. *Cardiovasc Diabetol*. 2014;13(1):49. doi:10.1186/1475-2840-13-49
5. Shi L, Ji Y, Jiang X, et al. Liraglutide attenuates high glucose-induced abnormal cell migration, proliferation, and apoptosis of vascular smooth muscle cells by activating the GLP-1 receptor, and inhibiting ERK1/2 and PI3K/Akt signaling pathways. *Cardiovasc Diabetol*. 2015;14(1):18. doi:10.1186/s12933-015-0177-4
6. Luo B, He Z, Huang S, et al. Long Non-coding RNA 554 promotes cardiac fibrosis via TGF-β1 pathway in mice following myocardial infarction. *Front Pharmacol*. 2020;11:585680-585680. doi:10.3389/fphar.2020.585680
7. Paneni F, Beckman JA, Creager MA, Cosentino F. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I. *Eur Heart J*. 2013;34(31):2436-2443. doi:10.1093/eurheartj/eht149
8. Takahashi H, Nomiyama T, Terawaki Y, et al. Combined treatment with DPP-4 inhibitor linagliptin and SGLT2 inhibitor empagliflozin attenuates neointima formation after vascular injury in diabetic mice. *Biochem Biophys Rep*. 2019;18:100640-100640. doi:10.1016/j.bbrep.2019.100640
9. Marso SP, Daniels GH, Brown-Frandsen K, et al; LEADER Steering Committee; LEADER Trial Investigators. Liraglutide and cardiovascular outcomes in type 2 diabetes. *N Engl J Med*. 2016;375(4):311-322. doi:10.1056/NEJMoa1603827
10. Tsai T-H, Lee C-H, Cheng C-I, et al. Liraglutide inhibits endothelial-to-mesenchymal transition and attenuates neointima formation after endovascular injury in streptozotocin-induced diabetic mice. *Cells*. 2019;8(6):589. doi:10.3390/cells8060589
11. Bretón-Romero R, Weisbrod RM, Feng B, et al. Liraglutide treatment reduces endothelial endoplasmic reticulum stress and insulin resistance in patients with diabetes mellitus. *J Am Heart Assoc*. 2018;7(18):e009379-e009379. doi:10.1161/JAHA.118.009379
12. Duan XJ, Zhang X, Li L-R, Zhang J-Y, Chen Y-P. MiR-200a and miR-200b restrain inflammation by targeting ORMDL3 to regulate the ERK/MMP-9 pathway in asthma. *Exp Lung Res*. 2020;46(9):321-331. doi:10.1080/01902148.2020.1778816
13. Guo C, Wang Y, Piao Y, Rao X, Yin D. Chrysophanol inhibits the progression of diabetic nephropathy via inactivation of TGF-β pathway. *Drug Des Devel Ther*. 2020;14:4951-4962. doi:10.2147/DDDT.S274191
14. Xie S, Ge F, Yao Y, et al. The aqueous extract of Lycopus lucidus Turcz exerts protective effects on podocytes injury of diabetic nephropathy via inhibiting TGF-β1 signal pathway. *Am J Transl Res*. 2019;11(9):5689-5702.
15. Ziller N, Kotolloshi R, Esmaeili M, et al. Sex differences in diabetes- and TGF-β1-induced renal damage. *Cells*. 2020;9(10):2236. doi:10.3390/cells9102236
16. Wang J, Yu M, Xu J, et al. Glucagon-like peptide-1 (GLP-1) mediates the protective effects of dipeptidyl peptidase IV inhibition on pulmonary hypertension. *J Biomed Sci*. 2019;26(1):6. doi:10.1186/s12929-019-0496-y
17. Lee J-H, Mellado-Gil JM, Bahn YJ, Pathy SM, Zhang YE, Rane SG. Protection from β-cell apoptosis by inhibition of TGF-β/Smad3 signaling. *Cell Death Dis*. 2020;11(3):184-184. doi:10.1038/s41419-020-2365-8
18. Buhrmann C, Yazdi M, Popper B, Kunnumakkara AB, Aggarwal BB, Shakibaei M. Induction of the epithelial-to-mesenchymal transition of human colorectal cancer by human TNF-β (lymphotoxin) and its reversal by resveratrol. *Nutrients*. 2019;11(3):704. doi:10.3390/nu11030704
19. Buhrmann C, Yazdi M, Popper B, et al. Evidence that TNF-β induces proliferation in colorectal cancer cells and resveratrol can down-modulate it. *Exp Biol Med (Maywood)*. 2019;244(1):1-12. doi:10.1177/1535370218824538
20. Boraska V, Zeggini E, Groves CJ, et al. Family-based analysis of tumor necrosis factor and lymphotoxin-alpha tag polymorphisms with type 1 diabetes in the population of South Croatia. *Hum Immunol*. 2009;70(3):195-199. doi:10.1016/j.humimm.2008.12.010