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Liraglutide improves myocardial fibrosis after myocardial infarction through inhibition of CTGF by activating cAMP in mice

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Abstract. – OBJECTIVE: To study the role and mechanism of liraglutide in myocardial fibrosis after myocardial infarction (MI).

MATERIALS AND METHODS: A total of 72 C57/BL male mice were randomly divided into sham operation group (Sham group), myocardial infarction group (MI group), and liraglutide intervention group (Lira group). The left anterior descending coronary artery (LAD) of the mice in MI group and Lira group was ligated to establish the MI model. One week after the operation, the mice in Lira group were intraperitoneally injected with 100 μg/kg of liraglutide once a day for 4 weeks. The mice in Sham group and MI group were injected with the equal volume of normal saline. At the 5th week after the operation, the cardiac morphologic indexes and cardiac function indexes were measured by echocardiography. After an ultrasound, the heart specimens of the mice were immediately harvested by thoracotomy, and histomorphological hematoxylin-eosin (HE) staining, collagen fiber Masson staining, and immunohistochemical staining were performed. The infarction zone and the non-infarction zone were isolated from another heart specimen; the cyclic adenosine monophosphate (cAMP) and hydroxyproline content were determined; and the expression levels of transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) were detected by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting.

RESULTS: Liraglutide improved the cardiac function of mice after myocardial infarction. Liraglutide improved the myocardial fibrosis in mice after myocardial infarction. Liraglutide increased cAMP in myocardial cells of mice after myocardial infarction. Liraglutide did not change the TGF-β1 expressions while reduced the CTGF expressions in infarct and non-infarct area of mice after myocardial infarction.

CONCLUSIONS: Liraglutide, through increasing the level of cAMP, could decrease the deposition of collagen fibers in myocardial tissues of mice after MI, reduce the degree of infiltration of collagen fibers in the infarction zone into the myocardium in the non-infarction zone and inhibit the adverse ventricular remodeling in the non-infarction zone, thus improving the cardiac function after MI.

Key Words:
Liraglutide, Fibrosis, Myocardial infarction, cAMP, TGF-β1, CTGF.

Introduction

Fibrosis plays an important role in the process of ventricular remodeling after myocardial infarction (MI)[1,2]. The heart repair in the infarct area after MI is mainly dependent on the tissue fibrosis in the infarct area. Good fibrotic scar repair helps maintain the complete shape and contractile function of the heart. According to clinical studies[3-5], MI patients that receive effective treatment and coronary perfusion is improved effectively, but the adverse ventricular remodeling affecting the cardiac function in the repair after MI is still an important issue worthy of attention. Scholars[6,7] have indicated that the progression and regulation of fibrosis in the infarction zone and non-infarction zone after MI are not the same, and dysregulation may occur, resulting in excessive fibrosis. In particular, secondary interstitial fibrosis of myocardium in the non-infarction zone will aggravate myocardial ischemia and hypoxia, increase ventricular stiffness and reduce ventricular

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compliance, resulting in systolic dysfunction, affecting ventricular output after MI and greatly increasing the incidence of heart failure[8]. Therefore, inhibiting myocardial fibrosis, especially that in the non-infarction zone, is an effective method to relieve left ventricular remodeling and improve cardiac function after MI.

As an important second messenger signaling molecule in cells, cyclic adenosine monophosphate (cAMP) plays an important role in regulating multiple functions and proliferation of cells[9]. Authors[10-12] have revealed that increasing the level of cAMP in target cells cannot only selectively inhibit the expression of connective tissue growth factor (CTGF) gene, but also inhibit the proliferation and collagen synthesis of lung fibroblasts and kidney fibroblasts. Liraglutide, a GLP-1 analog, is a novel hypoglycemic drug, whose activity is mediated *via* the specific interaction between it and GLP-1 receptor, thus increasing the level of cAMP[13,14]. Researches[15,16] have demonstrated that GLP-1 has a protective effect on myocardial ischemia-reperfusion injury in mice, but whether it can improve myocardial fibrosis after MI by increasing cAMP concentration remains unclear yet. In this investigation, the mouse model of MI was established to simulate the post-MI pathological process, and liraglutide was administered for intervention to observe its role and mechanism in myocardial fibrosis after MI.

Materials and Methods

Experimental Animals and Models

Male C57/BL mice, weighing 20-25 g, were from Jilin University Animal Center. Establishment of the mouse model of MI[17]: Mice were intraperitoneally injected with 3.3% chloral hydrate for anesthesia and fixed on the operating table. After tracheal intubation, the rodent ventilator was switched on. The mice were deprived of the left chest hair and subjected to routine disinfection. The thoracic cavity of the mice was entered through the intercostal space with the strongest cardiac impulse, and a self-made rib spreader was used to expose the heart. The pericardium was removed and the left anterior descending coronary artery (LAD) was located. LAD was ligated together with a small amount of myocardium using the 8/0 atraumatic suture. When the myocardium below the ligation point changed from red to white, the apical impulse was weakened, and the intraoperative electrocardiogram displayed the ST segment elevation in an arch shape, it was confirmed that the MI model was successfully made. The air in the thoracic cavity was drained and the chest wall was sutured with the 5/0 atraumatic suture. The mice were kept warm with blankets until they recovered from anesthesia.

This study was approved by the Animal Ethics Committee of The First Hospital of Jilin University Animal Center.

Animal Grouping and Drug Intervention

The experimental mice were randomly divided into sham operation group (Sham group), myocardial infarction group (MI group), and liraglutide intervention group (Lira group), 24 mice in each group. The mice in Sham group were subjected to thoracotomy and their blood vessels were threaded with the suture without being ligated. The remaining operations were the same. Drug intervention: One week after the operation, the mice in Lira group were intraperitoneally injected with 100 μg/kg of liraglutide once a day for 4 weeks, and the mice in Sham group and MI group were injected with the equal volume of normal saline.

Echocardiography

At the 5th week after the operation, 10 mice were randomly selected from each group. They were anesthetized with 3% isoflurane and fixed on an ultrasound test table, accompanied by continuous inhalation of 1.5% isoflurane to maintain anesthesia. The mice were deprived of left chest hair and then smeared with a small amount of ultrasound coupler. The echocardiography examination was performed with a Canada VEVO 2100 (Toronto, Canada) high-frequency animal ultrasound system for the mice.

Hematoxylin-Eosin (HE) Staining

The chest was quickly opened to obtain the heart specimen. Then, the specimen was fixed in 4% paraformaldehyde for 30 min, dehydrated, transparentized, and embedded into paraffin to prepare the paraffin sections. Paraffin sections were dewaxed for 10 min, dehydrated with gradient alcohol step by step, rinsed with phosphate-buffered saline (PBS) for 5 min, immersed in hematoxylin staining solution for 10 min, and washed with tap water, followed by differentiation with 1% hydrochloric acid alcohol for 10 s, staining with eosin staining solution for 5 min, and rinsing with tap water. After that, stained sections were dehydrated step by step using alcohol from a low concentration to a high concentration

and transparentized in xylene for 10 min. Finally, sections were covered with the coverslip, and sealed with resin.

Masson Staining

Paraffin sections were dewaxed with xylene for 10 min, dehydrated with gradient alcohol, stained with Masson compound staining solution for 5 min, differentiated with 5% phosphotungstic acid for 5 min, and soaked in aniline blue solution for 5 min. Then, stained sections were dehydrated step by step with alcohol and transparentized with xylene for 10 min. Finally, sections were covered with the coverslip and sealed with resin.

Immunohistochemical Staining

After paraffin sections were dewaxed with xylene for 10 min and dehydrated with gradient alcohol, 0.01 mmol/L citric acid buffer was added to completely submerge the sections, heated and boiled for 15 min. Then, sections were taken out and immersed in 0.03% hydrogen peroxide-methanol solution for 20 min. The antigen was sealed with sealing solution at room temperature for 10 min. The range was marked on the sections, and the diluted primary antibody was added dropwise for incubation at 4°C overnight. The horseradish peroxidase-labeled secondary antibody immunoglobulin G (IgG) polymer was added dropwise for incubation at room temperature for 30 min. After diaminobenzidine (DAB) developing solution (Beyotime, Shanghai, China) was added dropwise, sections were observed under a microscope. The staining time was controlled, and the color development reaction was terminated when there were brown-yellow positive signals. Sections were added dropwise with hematoxylin for nucleus counterstaining for 40 s. Finally, sections were covered with the coverslip and sealed with resin.

Determination of Content of cAMP and Hydroxyproline in Collagen Fibers

Heart tissues in infarction zone and non-infarction zone were isolated, accurately weighed and placed into a test tube. The absorbance value of specimens in each group was detected in strict accordance with instructions of the cAMP and hydroxyproline assay kits.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Heart tissues in infarction zone and non-infarction zone were isolated. Ribonucleic acid (RNA) was extracted from the specimen using the TRIzol method (Invitrogen, Carlsbad, CA, USA), and the RNA concentration was detected using a spectrophotometer. An equal amount of RNA was taken from the specimen in each tube and reversely transcribed into complementary DNA (cDNA). Products were amplified *via* PCR, followed by electrophoresis for 25 min. After that, the gel was taken out, stained with ethidium bromide (0.5 μg/mL) for 15 min and scanned using an ultraviolet detector. Finally, results were analyzed. Primer sequences used in this study were as follows: TGF-β1, F: 5'-GAGAGCCCTGGATACCAACATCTG-3', R: 5'-GTGTGTCCAGGCTCCAAATGTAG-3'; CTGF, F: 5'-GGGTTACCAATGACAACGCATTC-3', R: 5'-CTCGGTATGTCTTCATGCTGGTG-3'. U6: Forward: 5'-CTCGCTTCGGCAGCACA-3', Reverse: 5'-AACGCTTCACGAATTTGCGT-3'

Western Blotting

Heart tissues in infarction zone and non-infarction zone were isolated, cut into pieces, smashed in a mortar and added with radioimmunoprecipitation assay (RIPA) lysis solution (Beyotime, Shanghai, China) for further tissue homogenization. The supernatant was retained for standby application. The protein concentration in the specimen was determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). 50 μg total protein was taken from the specimen in each group for electrophoresis under the constant pressure of 115 V for 135 min. After the protein was transferred into a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and sealed with sealing solution at room temperature on a shaker for 1 h, the PVDF membrane was placed into the diluted primary antibody solution for incubation at 4°C overnight, and immersed in the secondary antibody solution for incubation on the shaker at room temperature for 2.5 h. An appropriate amount of enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added for reaction for 2 min, followed by image development and observation.

Statistical Analysis

All data were presented as mean ± standard deviation ($\bar{x} \pm s$). The group *t*-test was used for the intergroup comparison of means. Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for analysis. $p<0.05$ suggested that the difference was statistically significant.

Results

Lira Improved the Cardiac Function of Mice After MI

At 5 weeks after the operation, an echocardiogram showed that the left ventricular systolic and diastolic diameters in MI group and Lira group were significantly increased compared with those in Sham group, but the anterior and posterior ventricular wall motion was significantly reduced compared with that in Sham group (Figure 1A). In MI group and Lira group, the cardiac function indexes, ejection fraction (EF%), and fractional shortening (FS%) were decreased compared with those in Sham group (Figure 1B). The cardiac morphology indexes of the left ventricle, Aws, Pws, Awd, Pwd, left ventricular end systolic diameter (LVESD) and left ventricular end diastolic diameter (LVEDD), in Lira group had no statistically significant differences compared with those in MI group. The cardiac function indexes, EF% and FS% and stroke volume (SV), in Lira group were superior to those in MI group (Figure 1C).

Lira Improved the Myocardial Fibrosis in Mice After MI

According to the pathological examination *via* HE staining, myocardial tissues in Sham group were arranged orderly with clear texture structure. Necrosis occurred in a large number of myocardial tissues in MI group and Lira group, myocardial tissues were ruptured and curled with disordered texture, the left ventricular cavity wall was significantly thinned, and the ventricular cavity was markedly enlarged (Figure 2A). Collagen fibers displayed blue color in Masson staining. No evident collagen fibrous proliferation was observed in Sham group, while there was a large amount of collagen fibrous proliferation in the infarction zone of left ventricle infiltrating into the non-infarction zone in MI group and Lira group. 4 weeks after the intervention with Lira, the degree of infiltration of collagen fibers into the non-infarction zone in Lira group was reduced compared with that in MI group (Figure 2B). The determination of hydroxyproline content manifested that the hydroxyproline content in the infarction zone in MI group and Lira group was significantly increased compared to that in Sham group. The

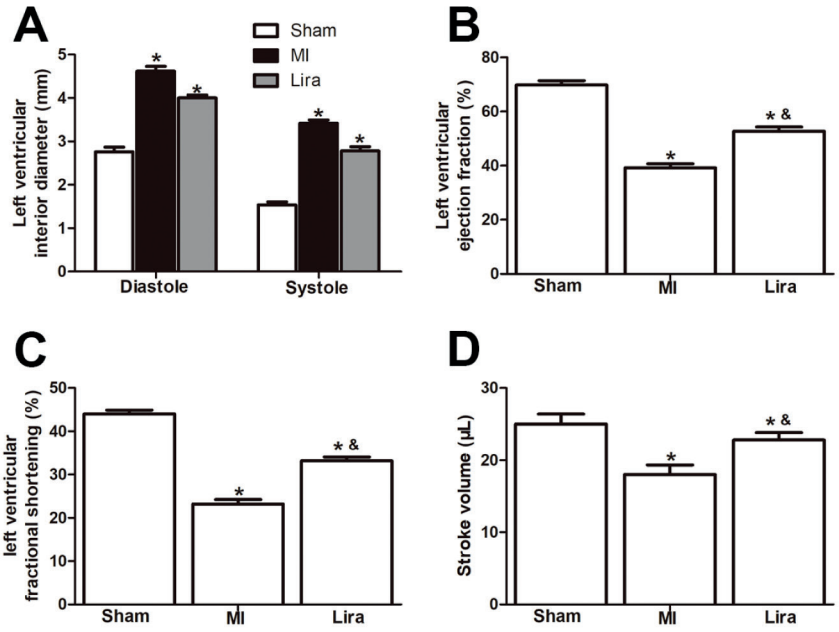

Figure 1. Liraglutide improved the cardiac function of mice after myocardial infarction. ***A,*** Analysis of left ventricular internal diameter in systole and diastole. ***B,*** Analysis of left ventricular ejection fraction. ***C,*** Analysis of left ventricular fractional shortening. ***D,*** Analysis of left ventricular stroke volume. **p*<0.05 *vs.* Sham group, &*p*<0.05 *vs.* MI group.

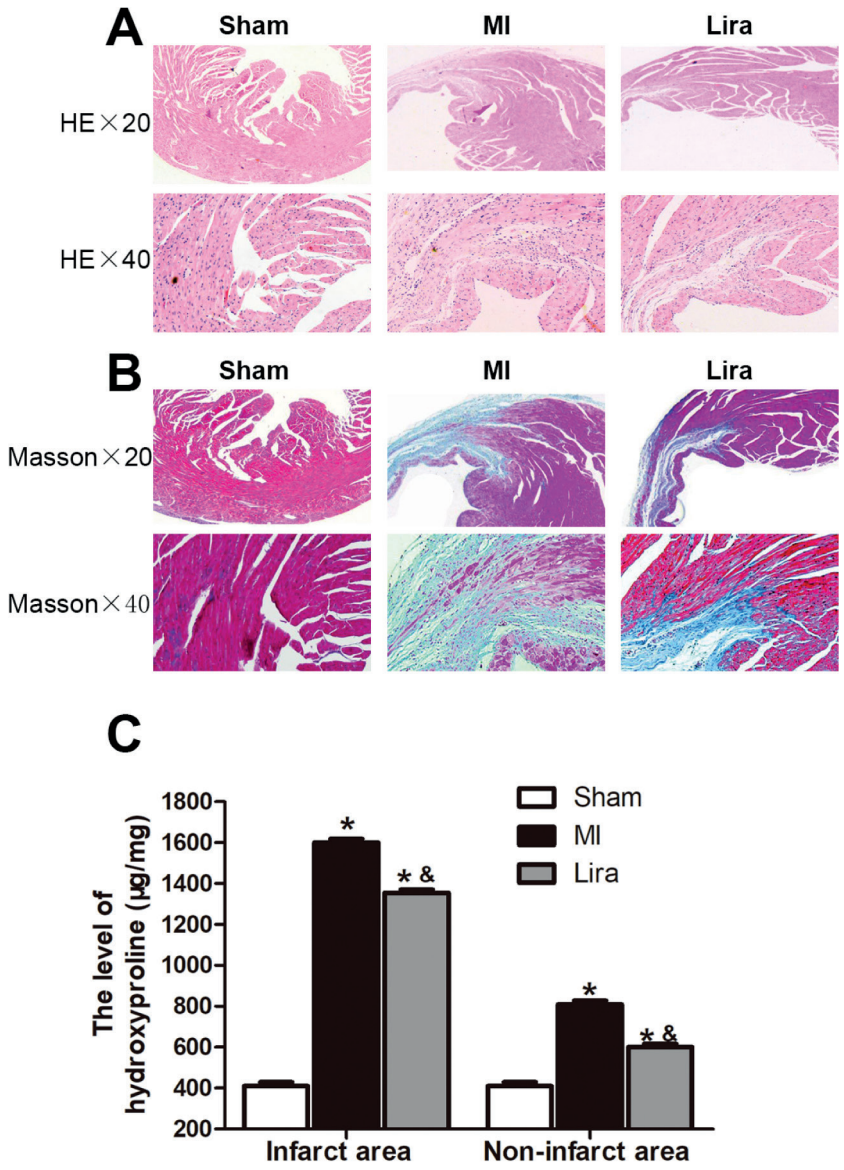

Figure 2. Liraglutide improved the myocardial fibrosis in mice after myocardial infarction. ***A,*** Representative images of HE staining. ***B,*** Representative images of Masson staining. ***C,*** Analysis of left level of hydroxyproline in heart tissue. **p*<0.05 *vs.* Sham group, &*p*<0.05 *vs.* MI group.

hydroxyproline content in the non-infarction zone in MI group and Lira group was higher than that in Sham group. After 4 weeks of intervention with Lira, the hydroxyproline content in both infarction zone and non-infarction zone in Lira group was lower than that in MI group (Figure 2C).

Lira Increased cAMP in Myocardial Cells of Mice After MI

The determination of cAMP content displayed that the cAMP content in myocardial cells in MI group was significantly decreased compared with that in Sham group. After 4 weeks of intervention with Lira, the cAMP content in myocardial cells in Lira group was significantly increased compared to that in MI group (Figure 3).

Lira Did Not Change the TGF-β1 Expressions in Infarct and Non-Infarct Area of Mice After MI

The positive immunohistochemical reaction products were brown yellow particles. No positive products were markedly expressed in Sham group, and a large number of TGF-β1 positive re-

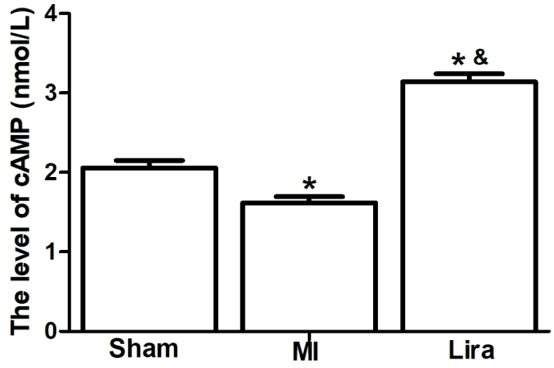

Figure 3. Liraglutide increased cAMP in myocardial cells of mice after myocardial infarction. *p<0.05 *vs.* Sham group, &p<0.05 *vs.* MI group.

action products could be seen in the infarct area in MI group and Lira group, and TGF-β1 positive reaction products were also detected in the non-infarct area. There were no statistically significant differences in the expression levels in the infarct area and non-infarct area between MI group and Lira group (Figure 4A). Results of RT-PCR demonstrated that the mRNA expression levels of TGF-β1 in the infarct area and non-infarct area in MI group and Lira group were remarkably increased compared with those in Sham group. After the intervention with Lira, the mRNA expression levels of TGF-β1 in the infarct area and non-infarct area in Lira group had no statistically significant differences compared with those in MI group (Figure 4B). Results of Western blotting manifested that the protein expression levels of TGF-β1 in the infarct area and non-infarct area in MI group and Lira group were remarkably increased compared with those in Sham group. After the intervention with Lira, the protein expression levels of TGF-β1 in the infarct area and non-infarct area in Lira group had no statistically significant differences compared with those in MI group (Figure 4C, 4D).

Lira Reduced the CTGF Expressions in Infarct And Non-Infarct Area of Mice After MI

The positive CTGF immunohistochemical reaction products were brown yellow particles. No positive products were significantly expressed in Sham group, and massive CTGF positive reaction products could be seen in the infarct area in MI group and Lira group, and CTGF positive reaction products were also detected in the non-infarct area. After the intervention with Lira in Lira group, the CTGF positive reaction products in the border area and non-infarct area were decreased compared with those in MI group (Figure 5A). According to results of RT-PCR, MI group and Lira group had increased mRNA expression levels of CTGF in the infarct area and non-infarct area compared with Sham group. The mRNA expression levels of CTGF in the infarct area and non-infarct area in Lira group were lower than those in MI group (Figure 5B). Results of Western blotting manifested that the protein expression levels of CTGF in the infarct area and non-infarct area were remarkably increased in MI group and Lira group compared with those in Sham group. After the intervention with Lira for 4 weeks, the protein expression levels of CTGF in the infarct area and non-infarct area in Lira group were lower than those in MI group (Figure 5C, 5D).

Discussion

Myocardial fibrosis is a common pathological manifestation of a variety of heart diseases developing to a certain stage, which is the most characteristic structural change and plays an important role in ventricular remodeling[1,2]. Fibrotic scar repair in the heart injury area after MI is of important and irreplaceable significance in keeping the integrity of heart structure, maintaining the heart pump function, and delivering the blood to each organ effectively[18,19]. The regulation of fibrosis after MI is complex, and the fibrotic process and regulation are different in the infarction zone and non-infarction zone of the heart tissues, both of which are likely to be disordered[6]. The fibrotic scar after MI is mainly composed of type I collagen fibers[20]. Type I collagen fibers are very hard, whose excessive deposition will affect the production and transmission of forces among myocardial cells, increase ventricular stiffness, reduce ventricular compliance and even make part of the ventricular wall lose contractility, ultimately leading to refractory heart failure. Studies have demonstrated that after the fibrotic scar repair in the infarction zone after MI, the fibrosis process of heart tissues does not stop but continues, and the non-infarction zone is affected in different degrees throughout the fibrosis process[21]. Therefore, regulating the fibrotic process after MI properly, avoiding fiber remodeling detrimental to cardiac function in the non-infarction zone as far as possible and protecting non-ischemic myocardial cells have positive significance in maintenance and protection of the heart function after MI, provided that rea-

sonable fibrotic scar repair in the infarction zone is ensured as far as possible.

TGF-β1, as a potent pro-fibrogenic factor, is widely involved in the process of fibrosis, whose pro-fibrogenic effect is realized mainly through the activation of downstream signaling molecule CTGF[22,23]. As an important downstream signal molecule of TGF-β1, CTGF is directly involved in the fibrosis process. Studies have revealed that TGF-β1 only plays a role in the initial stage of fibrosis, whose expression level gradually declines with time. The continuous progression of fibrosis is closely related to the continuous expression of CTGF[21]. It was found in the animal experiments that the expression of TGF-β1 reaches the peak at 1 week after MI and then gradually declines, while the expression of CTGF is up-regulated continuously after MI until 16 weeks after injury[21]. Therefore, the treatment based on TGF-β1 alone can only block the initiation of fibrosis, but once CTGF is activated by TGF-β1, both CTGF expression and fibrosis process will continue even if there is no sustained stimulation of TGF-β1. Therefore, CTGF is more specific than TGF-β1 in the selection of the anti-fibrotic target, and selectively inhibiting the CTGF expression or reducing its activity can not only effectively inhibit the progression of myocardial fibrosis, but also retain the normal biological function of TGF-β1.

cAMP is an important second messenger in cells, which plays an important role in regulating multiple functions and proliferation of cells. According to studies[10,11], increasing the cAMP level in cells can inhibit the CTGF gene expression and collagen synthesis. In other studies[10,12], it was also found that the cell proliferation is inhibited after cAMP intervenes in lung fibroblasts and kidney fibroblasts[10,12]. At the same time, clinical resear-

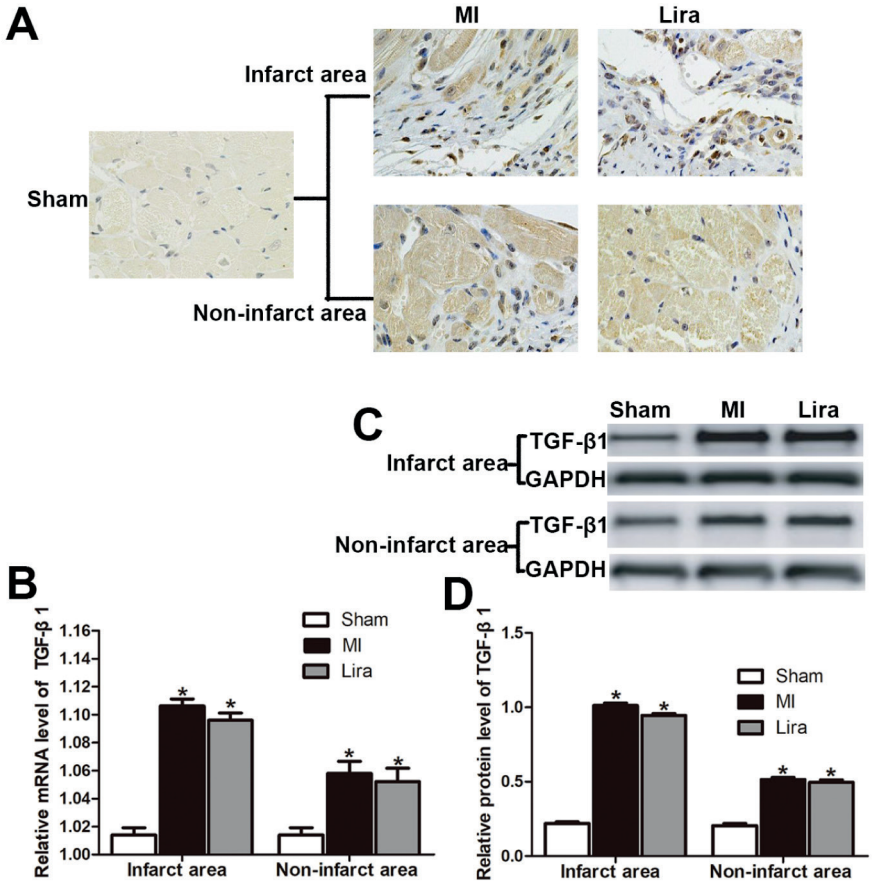

Figure 5. Liraglutide reduced the CTGF expressions in infarct and non-infarct area of mice after myocardial infarction. ***A,*** Representative images of immunohistochemistry of CTGF. ***B,*** RT-PCR analysis reveals the expression of CTGF mRNA. ***C,*** Western blots analysis reveals the expression of CTGF. ***D,*** Semi-quantitative analysis of CTGF. *p<0.05 *vs.* Sham group, &p<0.05 *vs.* MI group.

ch manifested that the cAMP level is significantly decreased, and CTGF is highly expressed in atherosclerotic lesions[24]. Therefore, it is speculated that cAMP may exert a regulatory effect on myocardial fibrosis after MI. As a kind of novel hypoglycemic drug, Lira can increase the cAMP level, so it was used as an intervention drug in this study. It is extremely important to select the time point for anti-fibrosis treatment after MI, and too early or too late intervention with anti-fibrosis drugs cannot achieve the goal of reasonable and effective anti-fibrosis. Studies have demonstrated that the time to peak for collagen deposition after MI is inconsistent in various animals. It takes about 1 week for the normal scar formation in mice. Therefore, the drug intervention began at 1 week after MI in mice in this work, so as to avoid the influence on normal scar formation in the infarction zone.

Lira, a GLP-1 analog, whose effects have been already demonstrated by some large double-blind trials (LEADER). LEADER is a phase 3B randomized, double-blind clinical trial to evaluate the cardiovascular safety of Lira in patients with type 2 diabetes (T2DM) at heightened risk for cardiovascular complications. Another LEADER trial has shown that patients with T2DM who received treatment of Lira had lower rates of cardiovascular events and death from any cause than those in the placebo group[25,26].

In MI group in this report, the anterior and posterior heart walls of mice were thinned, the heart was enlarged, cardiac function indexes (EF%, FS%, and SV) were significantly reduced compared with those in Sham group, the cAMP level was significantly reduced, and CTGF protein and mRNA expressions and hydroxyproline content in the infarction zone and non-infarction zone were markedly increased compared with those in Sham group. Compared with those in MI group, the cAMP level was increased significantly, CTGF protein and mRNA expression levels in the infarction zone and non-infarction zone were down-regulated in different degrees, the hydroxyproline content was reduced, cardiac function indexes (EF%, FS%, and SV) were improved in different degrees in Lira group after Lira intervention for 4 weeks. However, the TGF-β1 mRNA expression levels in the infarction zone and non-infarction zone had no statistically significant differences between MI group and Lira group. Therefore, it is believed that Lira can inhibit the expression of CTGF *via* cAMP, which can improve myocardial fibrosis after MI, especially fibrosis in the non-infarction zone, without affecting the expression of its upstream signal molecule TGF-β1.

Conclusions

We found that liraglutide, through increasing the level of cAMP, inhibited the expression of CTGF and decreased the deposition of collagen fibers in myocardial tissues of mice after MI. Lira can also attenuate the transition of collagen fibers from the infarcted zone to the non-infarction myocardium and inhibit the adverse ventricular remodeling in the non-infarction zone, thus improving the cardiac function after MI.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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