Incremental effect of liraglutide on traditional insulin injections in rats with type 2 diabetes mellitus by maintaining glycolipid metabolism and cardiovascular function

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Abstract. Type 2 diabetes mellitus (T2DM) is characterized by chronic hyperglycemia, damaged insulin secretion and insulin resistance with high morbidity and mortality. Liraglutide (liragl) and insulin are effective hypoglycemic agents used in T2DM treatment. The potential effect of liragl in combination with insulin on T2DM remains unclear. The aim of the current study was to explore effects of liragl combined with insulin on glycolipid metabolism and cardiovascular function in rats with diabetes. A diabetes model was established in Sprague Dawley rats exposed to a high calorie and high sugar diet in conjunction with intraperitoneal injections of streptozotocin. Results indicated that liragl or insulin used alone decreased glucose and elevated insulin and c-peptide levels. However, their combination revealed greater effects. A significant increase in high-density lipoprotein cholesterol levels along with a decrease in total cholesterol, triglycerides and low-density lipoprotein cholesterol were observed in liragl- and insulin-treated rats compared with STZ-induced diabetes rats. Furthermore, co-administration of liragl and insulin significantly decreased sterol regulatory element-binding protein 1 levels and increased adenosine 5'-monophosphate kinase-α1 and carnitine palmitoyltransferase 1 expression. Combining liragl with insulin reduced myocardial hypertrophy level and gaps between cardiomyocytes compared with liragl or insulin treatment alone. Caspase-3 expression was significantly decreased by combination treatment of liragl and insulin. Oxidative damage was significantly decreased by co-administration of liragl and insulin through enhancing superoxide dismutase expression and reducing malondialdehyde. Furthermore, combination of liragl and insulin significantly reduced myocardial enzyme expression, including myoglobin, creatine kinase-muscle/brain and cardiac troponin I. In summary, the current study demonstrated synergistic effects of liragl and insulin injections on a T2DM rat model by maintaining glycolipid metabolism and cardiovascular function.

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Abbreviations: TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; T2DM, type 2 diabetes mellitus; liragl, liraglutide

Key words: liraglutide, type 2 diabetes mellitus, insulin, glycolipid metabolism, cardiovascular function

Introduction

Type 2 diabetes mellitus (T2DM), a glucose-, lipid-, protein- and water-electrolyte metabolic disorder, risks micro- and macrovascular damage (1). T2DM is associated with various chronic complications, which may result in high rates of disability and mortality (2). Major features of diabetes are impaired insulin secretion and insulin resistance (3). Diabetes is developing into a global health problem that threatens human health. Treatment has improved over the last decades, but therapeutic effects remain limited (4,5).

Insulin is used in the clinic to improve utilization of glucose and accelerate anaerobic glycolysis and aerobic oxidation of glucose, thereby reducing blood sugar levels (6). However, insulin treatment has side effects, including hypoglycemic shock, insulin resistance, local reactions of subcutaneous scleroma and fat atrophy (7). A drug reducing insulin-associated side effects is needed. The synthetic glucagon-like peptide-1 (GLP-1) receptor agonist, liraglutide (liragl), shares 97% homology with the structure of human native GLP-1 (8). Numerous studies have demonstrated that liragl increases insulin secretion and inhibits glucagon secretion (9,10). A long circulating half-life with few side effects make liragl an ideal long-acting antidiabetic drug (11). Co-administration of insulin and liragl may describe a novel therapy for T2DM.

Owing to interactions between lipid metabolic disorder and hyperglycemia, glycolipid metabolic disorder is becoming a major factor in T2DM and metabolic syndrome (12). In addition, glycolipid metabolic disorder contributes to diabetes and its associated complications, including cardiovascular diseases (13,14). A previous study has indicated that severe chronic vascular disease (CVD) is a major cause of co-morbidity and mortality in patients with T2DM (15). Cardiovascular disease has been identified as a threat to patients with diabetes, as the association between high blood glucose and cardiovascular disease has been confirmed, and

many trials have tested the hypothesis that glucose normalization should prevent vascular injury (16,17). Previous studies have indicated that patients with diabetes and cardiovascular diseases have increased mortality rates compared with patients without cardiovascular diseases (18,19). The main challenge in the successful management of T2DM is not only to control blood sugar levels, but also to reduce glycolipid metabolic disorders and cardiac damage.

The current study investigated whether liragl may reduce insulin-induced side effects in patients with diabetes, including glycolipid metabolic disorders and cardiac injury. The results demonstrated that co-administration of insulin and liragl may control blood sugar levels, restore the glycolipid metabolic balance and alleviate cardiac injury.

Materials and methods

Animals and ethics. A total of 40 adult male Sprague Dawley rats (age, 4 weeks; weight 220-250 g) were purchased from the Experimental Animal Center of Hebei Medical University (Shijiazhuang, China) and housed in a controlled environment at 25±3˚C in 60% humidity, in a 12-h light/dark cycle with free access to food and water. All experimental protocols were approved by the Committee for Laboratory Animal Care and Use of the Cangzhou Central Hospital (Cangzhou, China).

Induction of T2DM. STZ-induced model rats were exposed to high-fat diets (77% regular diet, 15% lard oil, 5% white sugar, 2% cholesterol, 0.25% sodium cholate, and 0.75% salt) for 4 weeks prior to receiving two intraperitoneal streptozotocin (STZ) injections (60 mg/kg) within 72 h. Rats were fed with the high-fat diets for a further 2 weeks. Rats with blood glucose level ≥11.1 mmol/l were considered as diabetic and selected for subsequent experiments. Healthy rats fed a normal diet were assigned as the control group (n=8) and diabetic rats were randomly divided into four groups (n=8 per group): STZ, Liragl, Insulin and Insulin + Liragl. Liragl group was treated with liragl (3 mg/day) by hypodermic injection in the abdomen; Insulin group was treated with insulin (50 U/day) by hypodermic injection in the abdomen and Insulin + Liragl group was treated with liragl (3 mg/day) and insulin (50 U/day) by hypodermic injection in abdomen. Treatment continued for 4 weeks. During this period, rats in healthy control group were fed with normal chow diet and rats with diabetes continued high-fat diet. All rats were sacrificed by cervical dislocation for subsequent experiments.

Blood-measured parameters. Rats who were deprived of food overnight for 12 h were sacrificed by cervical dislocation and blood was collected from the orbital sinus. Following centrifugation at 3,000 x g for 15 min at 4˚C, serum was collected for measurement of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) using a Hitachi 912 photometric chemistry analyzer (Hitachi, Ltd., Tokyo, Japan). The blood glucose concentration in the fasting state was measured using a blood glucose measurement kit (Roche Diagnostics, Basel, Switzerland), utilizing the glucose dehydrogenase method as previously described (20). Radioimmunoassays (cat. no. NEX133001KT; PerkinElmer Inc., Krakow, Poland) were performed to assess fasting insulin and C-peptide as described elsewhere (21).

Western blot assays. Hepatic and myocardial tissues were isolated from rats and homogenized separately on ice using a 10X RIPA buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing 1% phenylmethylsulfonyl fluoride. Homogenized samples were washed with ice-cold PBS and centrifuged at 10,000 x g for 15 min at 4˚C. The supernatant was collected and the protein concentration was determined using a BCA protein assay kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Proteins were separated using 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 5% skimmed milk at room temperature for 2 h, membranes loaded with hepatic proteins were incubated with primary antibodies at 37˚C for 4 h: Rabbit anti-adenosine 5'-monophosphate kinase-α1 (AMPKα1) (1:1,000; cat. no. ab3759), rabbit anti-carnitine palmitoyltransferase 1 (CPT-1) (1:5,000; cat. no. ab198494), rabbit anti-sterol regulatory element-binding protein 1 (SREBP-1c) (1:2,000; cat. no. ab28481) and rabbit anti-GAPDH (1:1,000, cat. no. ab9485) (all Abcam, Cambridge, UK). Membranes loaded with myocardial proteins were incubated with rabbit anti- myoglobin (Mb) (1:2,500; cat. no. ab77232), rabbit anti-creatine kinase-muscle/brain (CK-MB; 1:1,000; ab31832), rabbit anti-cardiac troponin I (cTnI; 1:1,000; ab10231) and rabbit anti-GAPDH (1:2,500) (all Abcam) at 4˚C overnight. Membranes were washed with Tris-buffered saline and Tween-20 three times and incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000; cat. no. ab181658; Abcam) for 1 h at room temperature. Proteins were visualized using enhanced chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Analysis was performed using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

Histopathological examination. Formalin fixed and paraffin-embedded heart tissues were fixed with 10% neutral buffered formalin at 4˚C for 4 h. Then tissues were cut into 4-μm-thick slices. All samples were stained with hematoxylin and eosin (H&E) at 4˚C for 2 h. Histopathological characteristics of hearts were observed using light microscopy (magnification, x400).

Immunohistochemistry. Caspase-3 in heart tissues was measured by immunohistochemistry. Paraffin sections of heart tissue was fixed with 10% neutral buffered formalin at 4˚C for 4 h. Then the tissues were deparaffinized in xylene, rehydrated in graded ethanol solutions and microwaved in sodium citrate buffer. Following cooling to room temperature, sections were incubated with 3% fresh H_2O_2, followed by blocking with 3% bovine serum albumin for 2 h (Thermo Fisher Scientific, Inc.) at 25˚C for 2 h. Sections were incubated with rabbit anti-caspase-3 (cat. no. #9662; 1:1,000; Cell Signaling Technology, Inc.) at 4˚C overnight. Following washing with Tris buffered saline for 5 min (repeated three times), all slides were incubated with secondary antibody (rabbit IgG; cat. no. #A32731; 1:200; Thermo Fisher Scientific, Inc.) for 30 min at

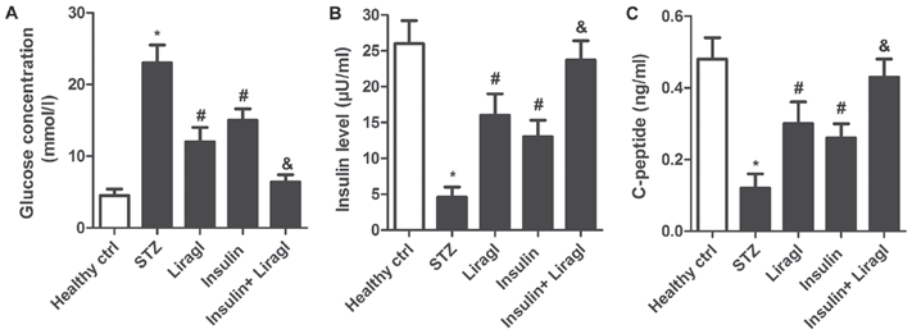

Figure 1. Liragl enhances hypoglycemic effects of insulin in type 2 diabetic rats. Sprague Dawley rats were randomly divided into groups: Healthy control group, healthy rats; STZ group, rats that received STZ injections to induce type 2 diabetes; Liragl group, diabetic rats treated with liragl; Insulin group, diabetic rats treated with insulin; Insulin + Liragl group, diabetic rats treated with insulin and liragl. (A) Blood glucose concentration measured in each group. (B) Insulin level and (C) C-peptide level detected using radioimmunoassays. *P<0.05 vs. healthy control group; #P<0.05 vs. STZ group; &P<0.05 vs. Insulin group. Liragl, liraglutide; STZ, streptozotocin.

37˚C. Sections were successively stained with 3,3'-diaminobenzidine for 5 min and counter-stained with hematoxylin for 30 sec at 4˚C. Sections were observed using a digital camera (under magnification, x400) following dehydrating, drying and mounting with neutral gum.

Evaluation of oxidative stress in serum. Malondialdehyde (MDA) in the serum was measured using an MDA Assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The content of MDA was determined by a thiobarbituric acid reaction. The absorbance was read at 532 nm by a spectrophotometer. For superoxide dismutase (SOD) detection, the cells were lysed using a cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and the lysates were centrifuged at 10,000 g at 4˚C for 5 min. The supernatant was collected for SOD analysis using a SOD kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The absorbance was read at 450 nm by a microplate reader. The mean value of each group was calculated as the percentage of the control value.

Statistics analysis. Data were analyzed with SPSS 19.0 (IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard deviation and a minimum of three repeats were performed for each experiment. Group statistical comparisons were assessed by one-way analysis of variance followed by Bonferroni's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Liragl enhances hypoglycemic effect of insulin in T2DM rats. To explore the hypoglycemic effect of combined liragl and insulin treatment in T2DM rats, fasting blood-glucose, insulin and c-peptide concentrations were measured. Fig. 1A demonstrated a significant decrease in the glucose concentration in the Insulin group and the Liragl group compared with the STZ group, while a decrease was also detected in the Insulin + Liragl group compared with the Liragl or Insulin groups. Insulin and c-peptide levels in the Insulin and Liragl groups were significantly lower compared with the Insulin + Liragl group (Fig. 1B and C). These results demonstrated that combined treatment with liragl and insulin enhanced the hypoglycemic effect.

Co-administration of liragl and insulin ameliorates disorder of lipid metabolism. To investigate the impact of liragl and insulin on lipid metabolism, TC, TG, LDL-C and HDL-C levels in serum were determined. As illustrated in Fig. 2A-C, an increase of TC, TG and LDL-C was observed in STZ rats, which was significantly decreased by insulin and liragl, with a further significant decrease observed for the combination treatment. In addition, an STZ-induced decrease in HDL-C was significantly reversed by insulin and liragl, with a further significant increase observed for the combination treatment (Fig. 2D). These results suggested that combination of liragl and insulin may better alleviate the disorder of lipid metabolism more effectively compared with liragl or insulin alone.

Combination of liragl and insulin regulates expression of proteins associated with lipid metabolism. To further investigate the regulatory role that liragl and insulin serve in lipid metabolism, AMPKα1, CPT-1 and SREBP-1c levels were determined using western blot assays. As presented in Fig. 3, liragl and insulin suppressed the decrease in AMPKα and CPT-1 levels and the increase in SREBP-1c induced by STZ. Co-administration of the two drugs produced greater effects compared with either drug alone.

Liragl enhances protective effects of insulin on diabetes-induced myocardial damage. To determine whether combination of liragl and insulin served a protective role in diabetes-induced myocardial injury, morphological histological features of heart tissues were measured using H&E staining. As illustrated in Fig. 4A, serious cardiomyocyte edemas and intercellular space dilatations were observed in intermuscular spaces in the STZ group compared with the healthy control group. These histopathological alterations were suppressed by liragl or insulin used alone and markedly inhibited by their combined treatment. In addition, the STZ-induced increase in caspase-3 expression could be repressed significantly by liragl or insulin alone, but a combination produced greater inhibitory effects (Fig. 4B).

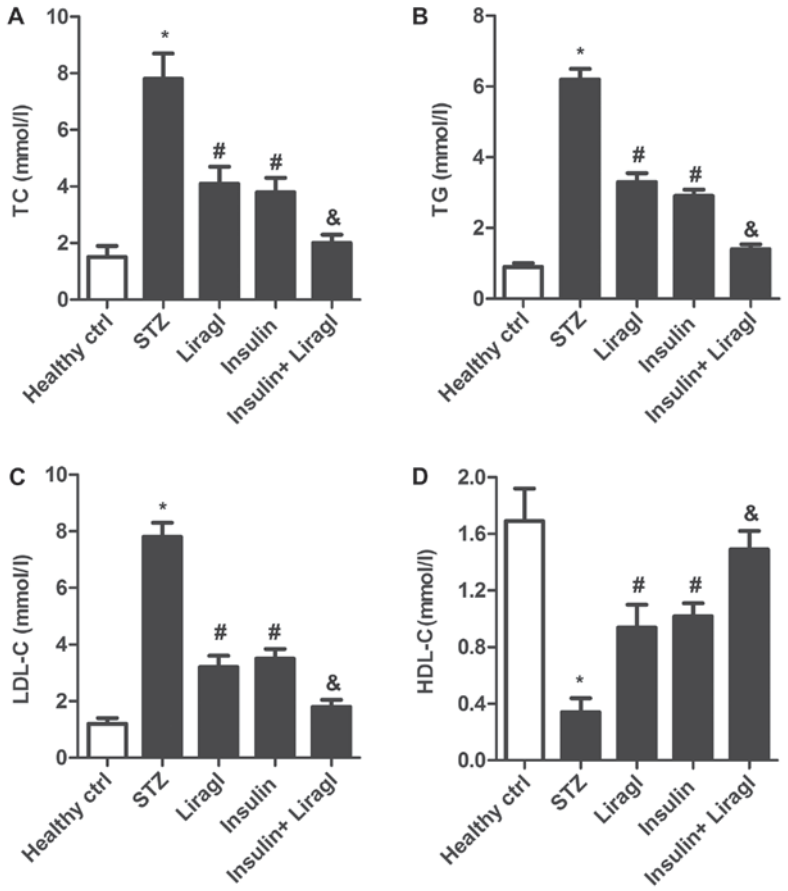

Figure 2. Co-administration of liragl and insulin ameliorates the disorder of lipid metabolism. Sprague Dawley rats were randomly divided into groups: Healthy control group, healthy rats; STZ group, rats that received STZ injections to induce type 2 diabetes; Liragl group, diabetic rats treated with liragl; Insulin group, diabetic rats treated with insulin; Insulin + Liragl group, diabetic rats treated with insulin and liragl. (A) TC, (B) TG, (C) LDL-C and (D) HDL-C levels. *P<0.05 vs. healthy control group; #P<0.05 vs. STZ group; &P<0.05 vs. Insulin group. Liragl, liraglutide; STZ, streptozotocin; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

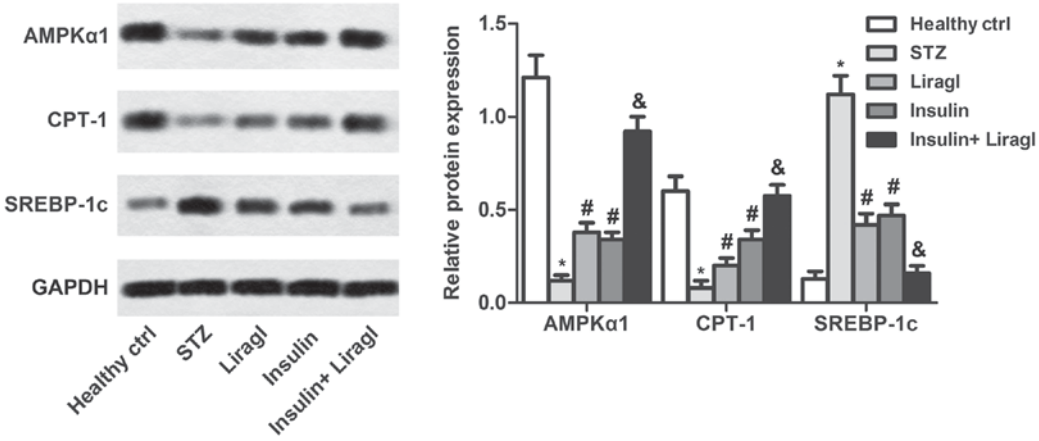

Figure 3. Combination of liragl and insulin regulates expressions of lipid metabolism associated proteins. Sprague Dawley rats were randomly divided into groups: Healthy control group, healthy rats; STZ group, rats that received STZ injections to induce type 2 diabetes; Liragl group, diabetic rats treated with liragl; Insulin group, diabetic rats treated with insulin; Insulin + Liragl group, diabetic rats treated with insulin and liragl. AMPKα1, CPT-1 and SREBP-1c expression determined using western blot analysis. *P<0.05 vs. healthy control group; #P<0.05 vs. STZ group; &P<0.05 vs. Insulin group. Liragl, liraglutide; STZ, streptozotocin; AMPKα1, adenosine 5'-monophosphate kinase-α1; CPT-1, carnitine palmitoyltransferase 1; SREBP-1c, sterol regulatory element-binding protein 1.

SOD, MDA, Mb, CK-MB and cTnI expression illustrated a similar trend. A decrease in SOD and an increase in MDA, Mb, CK-MB and cTnI expression as induced by STZ were significantly reversed by administration of liragl and insulin, with enhanced results when drugs were administered together (Fig. 4C and D). These results demonstrated that combination

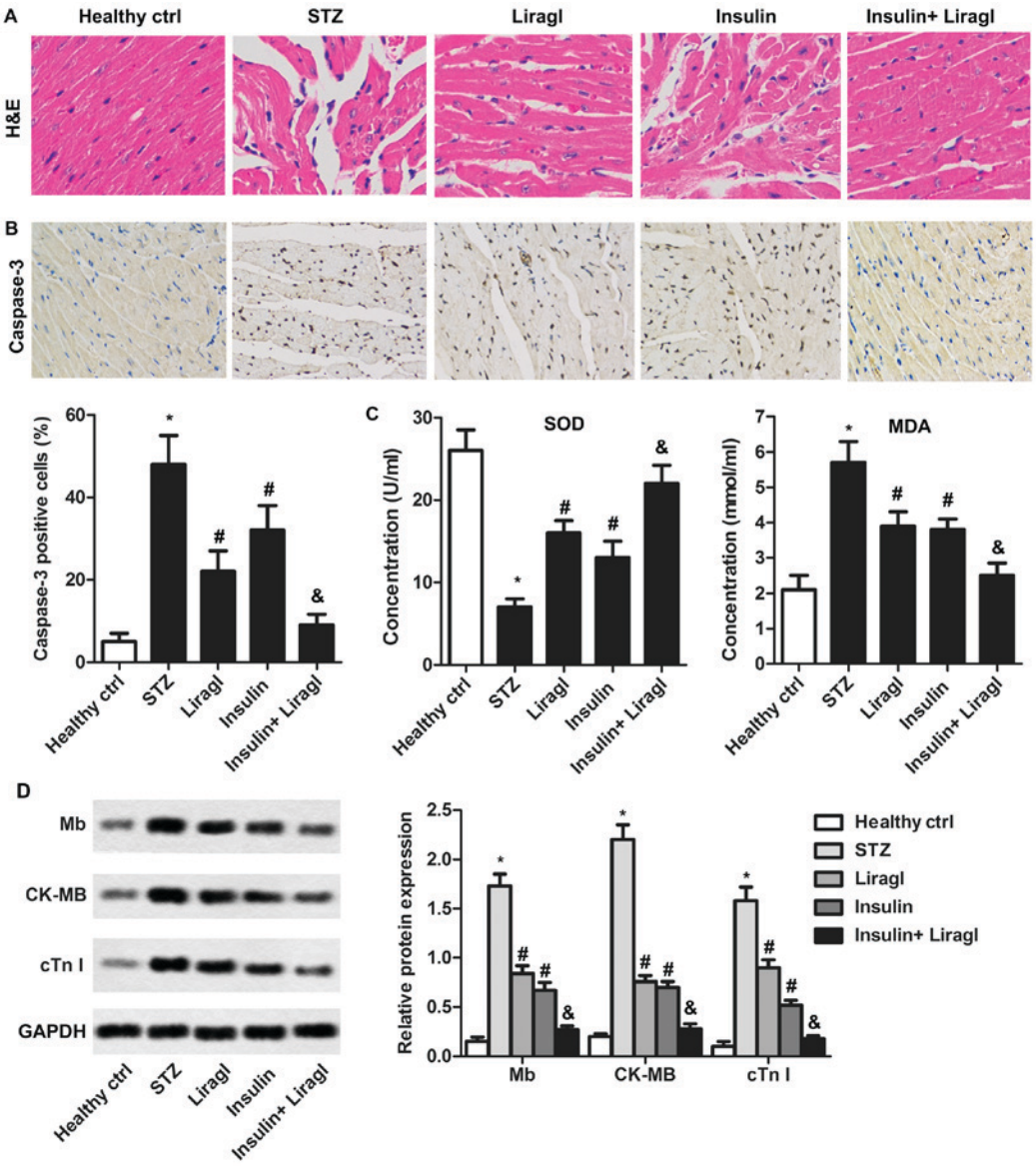

Figure 4. Liragl enhances protective effect of insulin on diabetes-induced myocardial damage. Sprague Dawley rats were randomly divided into groups: Healthy control group, healthy rats; STZ group, rats that received STZ injections to induce type 2 diabetes; Liragl group, diabetic rats treated with liragl; Insulin group, diabetic rats treated with insulin; Insulin + Liragl group, diabetic rats treated with insulin and liragl. (A) Morphological changes induced by diabetes measured using H&E staining. Magnification, x400. (B) The Caspase-3 expression was measured using immunohistochemistry assays. Magnification, x400 (C) SOD and MDA levels measured using commercial detection kits. (D) Mb, CK-MB and cTnI levels evaluated using western blot assays. *P<0.05 vs. healthy control group; $^{\#}$P<0.05 vs. STZ group; $^{\&}$P<0.05 vs. Insulin group. Liragl, liraglutide; STZ, streptozotocin; SOD, superoxide dismutase; H&E, hematoxylin and eosin; MDA, malondialdehyde; Mb, myoglobin; CK-MB, creatine kinase-muscle/brain; cTNI, cardiac troponin I.

of liragl and insulin may significantly alleviate myocardial injury and oxidative stress.

Discussion

Owing to the progressive nature of diabetes, many patients require multiple therapeutic approaches to control blood sugar levels (22). One major treatment is insulin. However, hypoglycemia, which depends on duration and dose of insulin treatment, limits application of insulin treatments (23).

A recent study indicated that liragl decreased glycated hemoglobin, enhanced insulin secretion, aided weight loss and rarely led to hypoglycemia (24). Based on these results, liragl was selected for combination treatment with insulin in the current study.

A major characteristic of diabetes is the disturbance of carbohydrate metabolism, which is caused by damaged islet β-cells (25). Islet β-cell injury is associated with increased blood glucose and decreased insulin and C-peptide levels (26). Kondo *et al* (27) described a retrospective cohort study to demonstrate that β-cell function was improved in early liragl treatment and increased C-peptide level in patients with T2DM. A previous study indicated that insulin therapy enhanced C-peptide expression over a short period (28). In the current study, liragl and insulin controlled blood sugar levels and increased insulin and C-peptide serum levels, and the co-administration of liragl and insulin resulted in more pronounced effects.

Patients with T2DM may exhibit serious damage of lipid dynamics, manifested as elevated TC, TG, LDL-C, decreased

HDL-C and excessive fat deposition in various tissues (29). According to Liu *et al* (30), liragl (1.2 mg/day) monotherapy exhibited significant lipid-lowering effects in patients with reduced levels of fasting blood glucose, glycated hemoglobin, body mass index, TG, TC and LDL-C following 24-week treatment. However, insulin resistance promoted small dense LDL and reduced HDL production. In the current study, combining liragl and insulin significantly elevated HDL-C levels and reduced TC, TG and LDL-C.

Myocardial damage induced by diabetes is a distinct entity, which is different from coronary heart disease (31). Increasing numbers of studies have demonstrated that liragl serves a positive role in cardiac functional recovery in patients with heart diseases (32,33). It is reported that primary endpoints of cardiac output, stroke volume and left ventricular contractile index were remarkably enhanced by liragl treatment for 7 days in patients with heart failure (34). For insulin, studies indicated beneficial effects of insulin on damaged cardiac tissue (35,36). Xing *et al* (37) reported activation of protein kinase B as a result of insulin-induced suppression of PH domain leucine-rich repeat-containing protein phosphatase 1 serving a vital role in cardioprotection. In the current study, serious cardiomyocyte edemas and intercellular space dilatations were alleviated by combination treatment of liragl and insulin. Co-administration of liragl and insulin significantly suppressed Mb, CK-MB and cTnI expression in heart tissue.

Oxidative stress and inflammation result in cardiomyocyte apoptosis in diabetic hearts, which eventually leads to cardiac dysfunction (38). Thus, suppressing apoptosis is extremely important. According to a published report, liragl treatment suppresses apoptosis of various cell types (39). Liragl improves recovery following central nervous system injuries through inhibiting apoptosis and elevating microtubulin acetylation and autophagy (40). In addition, in a previous study, an intramyocardial injection of nanoparticle-liragl promoted recovery of cardiac functions, alleviated infarct size and inhibited cardiomyocyte apoptosis at 4 weeks following injection (41). Insulin was reported to suppress cardiomyocytes apoptosis in rats with diabetic cardiomyopathy (42). In the current study, liragl combined with insulin suppressed apoptosis of cardiomyocytes via significantly decreased caspase-3 expression.

Increasing evidence indicates that aggregation of intermediate oxidation products may be a pathogenic factor of myocardial damage in diabetic rats (43). SOD, an important biological antioxidant, is involved in eliminating free radicals (44). MDA, a metabolite of lipid peroxidative damage, evaluates the extent of free radical-induced damage on cytomembranes (45). A previous study demonstrated that liragl treatment enhanced SOD and adiponectin levels in the liver, indicating antioxidative effects of liragl (46). Ramalingayya *et al* (47) observed that insulin (0.5 U/kg, intraperitoneal) attenuated doxorubicin-induced brain oxidative stress with an elevation in antioxidant defense systems. In the current study, combining liragl and insulin significantly increased SOD and decreased MDA levels in rats with T2DM.

In conclusion, the current study demonstrated that both liragl and insulin ameliorated diabetes and its complications, including glucose and lipid metabolism disorder and myocardial injury. However, combination treatment of insulin and liragl resulted in increased effects. Therefore, combination treatment of liragl and insulin may be considered as a potential therapeutic agent in diabetes treatment in the clinic.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QH analyzed and interpreted the data regarding the T2DM model and blood-measured parameters. CL was responsible for designing the study and drafting the manuscript. JRL and LZ performed the immunohistochemistry. FCH, DW and YJL performed the western blot and statistical analysis. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal experiments in this study were approved by the Animal Care and Research Committee of Cangzhou Central Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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