

Liraglutide, a glucagon-like peptide-1 analog, inhibits high glucose-induced oxidative stress and apoptosis in neonatal rat cardiomyocytes

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Abstract. Cardiomyocyte apoptosis serves an important role in diabetic cardiomyopathy. Liraglutide, a glucagon-like peptide-1 analog, has been indicated to exert a cardioprotective effect. However, the role of liraglutide on cardiomyocyte apoptosis in hyperglycemia is not fully understood. The aim of the current study was to assess whether liraglutide protects against high glucose (HG)-induced cardiomyocyte apoptosis *in vitro*. Sprague-Dawley neonatal rat cardiomyocytes were cultured in Dulbecco's modified Eagle's medium, supplemented with 5.5 or 25 mmol/l D-glucose or 5.5 mmol/l D-glucose + 19.5 mmol/l mannitol, in the presence or absence of liraglutide (10 or 100 nmol/l). Cell viability was assessed via an MTT assay and early apoptosis rates were assessed via flow cytometry. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content in cell supernatants were measured. Bcl-2 associated X (Bax), B-cell lymphoma-2 (Bcl-2) and cleaved/full caspase-3 protein levels were determined via western blotting. The results revealed that liraglutide effectively inhibited the HG-induced increase in early apoptosis and MDA content and markedly increased SOD activity. Furthermore, liraglutide markedly inhibited the HG-induced increase in Bax and cleaved caspase-3 protein expression, and upregulated the expression of Bcl-2. The present study demonstrated that liraglutide suppressed HG-induced oxidative stress and cardiomyocyte apoptosis. Thus, the anti-apoptotic actions of liraglutide may be attributable, in part, to the inhibition of Bax, the inhibition of caspase-3 activation and the upregulation of Bcl-2.

Introduction

Diabetic cardiomyopathy (DCM) is a serious complication of diabetes, which increases the mortality of patients (1). DCM is defined as left ventricular dysfunction that occurs independently of hypertension and coronary artery atherosclerosis and is a cause of heart failure in patients with diabetes (2). Increased oxidative stress and cardiomyocyte apoptosis have been implicated in the development of DCM (3,4). Therefore, inhibiting cardiomyocyte apoptosis is a key step in the prevention of DCM. Glucose-lowering agents that decrease the risk of major cardiovascular events would thus be considered important (5). Glucagon-like peptide-1 (GLP-1) is a 30-amino acid gut hormone that is secreted from intestinal endocrine L cells, which stimulates insulin secretion, inhibits glucagon secretion and inhibits gastric emptying, causing postprandial euglycemia and body weight reduction (6). Multiple GLP-1 analogs have been developed and one such agent, liraglutide, was approved for the treatment of type 2 diabetes and obesity (7). Growing evidence has indicated that GLP-1 analogs have the potential to reduce cardiac inflammation, limit infarct size and mitigate ischemic-reperfusion injury in animals with experimental myocardial infarction (MI) (8-10). Recently, several cardiovascular studies have documented the reduction of major adverse cardiovascular events and cardiovascular mortality in patients with type 2 diabetes or preexisting cardiovascular disease, via treatment with liraglutide and semaglutide (11,12). The beneficial effects exhibited by liraglutide and semaglutide may be associated with reductions in hemoglobin A1c, body weight, systolic blood pressure and lipoproteins (11-13). However, traditional atherogenic risk factor modifications alone cannot explain the overall benefits observed, indicating that additional mechanisms may occur (11-13). The favorable effects of liraglutide on oxidative stress and carotid atherosclerosis in patients with diabetes has been previously reported (14,15). Various studies have also revealed that liraglutide exhibits protective myocardial actions in diabetic animal models *in vivo* (16,17). However, the results of *in vivo* studies may have been influenced by many factors, including metabolic and environmental factors as well as the anti-atherosclerotic effect of liraglutide (15,17,18). Furthermore, few *in vitro* reports detail the role of liraglutide on cardiomyocytes in a high glucose (HG) state. Therefore, to assess the possible mechanism of

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liraglutide on myocardial protection in diabetes, the present study determined the effects of liraglutide on HG-induced oxidative stress and apoptosis in neonatal rat cardiomyocytes *in vitro*.

Materials and methods

Primary culture of rat myocytes. The animal protocol was reviewed and approved by the Laboratory Animal Ethical and Welfare Committee of Hebei Medical University (Shijiazhuang, China; approval no. IACUC-Hebmu-20160027). A total of 160 Sprague-Dawley (SD) rats (age, 3 days; weight, 8–10 g; 80 males and 80 females) were obtained from the Laboratory Animal Center of Hebei Medical University and used in the experiment immediately. Rat myocytes were prepared as previously described (19). Neonatal SD rats were euthanized using carbon dioxide (CO₂) and the flow rate displaced 20% of the chamber volume/minute. Rats were exposed to 50% CO₂ until they were euthanized, at which point they were decapitated. Rat ventricles were subsequently removed, minced and digested in PBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 0.1% trypsin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 0.04% type II collagenase (Invitrogen; Thermo Fisher Scientific, Inc.) eight to 10 times. Samples were centrifuged (320 x g, 37°C, 5 min) and suspended in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 5.5 mmol/l D-glucose. The suspension was maintained in DMEM for 2 h in a humidified atmosphere of 95% air and 5% CO₂ at 37°C, which was used to further increase the ratio of rat myocytes to non-cardiomyocytes. Unattached myocytes were plated at 1x10⁶ cells/cm² in the aforementioned medium supplemented with 0.1 mM bromodeoxyuridine (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 48 h. Myocytes were then placed in Lonza 12-725F UltraCULTURE serum-free medium (Lonza Walkersville, Inc., Walkersville, MD, USA) at 37°C for 24 h prior to experimentation. Rat myocytes were confirmed via morphological examination on a light microscope at a magnification of x400 and staining with an anti- α -sarcomeric actin (α -SMA) antibodies (cat. no. LM-10196R-FITC; dilution, 1:200; Sigma-Aldrich; Merck KGaA) overnight at 4°C. The α -SMA-positive cells were verified to be myocytes, ~95% of cells were identified as rat myocytes.

Drug treatments. Liraglutide, a GLP-1 analog, was obtained from Novo Nordisk Ltd. (Gatwick, UK). Liraglutide at a concentration of 10 and 100 nmol/l was selected according to previous studies (9,18) and cardiomyocyte viability determined in the present study (Fig. 1). When cardiomyocytes reached a confluence of 80%, cells were pre-incubated at 37°C in the presence or absence of 10 or 100 nmol/l liraglutide for 30 min. DMEM was then replaced with DMEM containing 5.5 mmol/l D-glucose [normal glucose (NG) group], 25 mmol/l D-glucose (HG group) or mannitol containing 5.5 mmol/l D-glucose and 19.5 mmol/l mannitol [osmotic control (OSM) group]. Myocytes of the HG group were further incubated at 37°C for 24 h in the presence of liraglutide (10 and 100 nmol/l; named the HG + 10 nM liraglutide and HG + 100 nM liraglutide groups, respectively).

Cell survival assay. Cell viability was assessed via an MTT assay (Sigma-Aldrich; Merck KGaA). Myocytes were plated at 1x10⁴ cells/well in 96-well plates and 20 μ l of 5 mg/ml MTT was added to each well and incubated for 4 h at 37°C. Samples were then solubilized with 150 μ l dimethyl sulfoxide. Absorbance was read at 490 nm. Each experiment was repeated three times and three independent experiments were performed.

Flow cytometry. A fluorescein isothiocyanate (FITC) Annexin-V apoptosis detection kit (BD Biosciences, San Jose, CA, USA) was used to detect apoptosis in neonatal rat cardiomyocytes following various treatments. Cardiomyocytes were washed in PBS three times and resuspended in 400 μ l of binding buffer with FITC Annexin V and propidium iodide (PI, 5 μ l of each). Cell suspensions were incubated for 15 min at room temperature in the dark and analyzed via flow cytometry within 1 h. FlowJo software (version 7.6; FlowJo LLC, Ashland, OR, USA) was used for data acquisition. Positive Annexin V-FITC and negative PI cells were identified as early apoptotic cells. Apoptosis rate was calculated as the number of early apoptotic cells relative to the total number of cells. Each experiment was repeated three times and three independent experiments were performed.

Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content. Following cell treatments, the supernatant was collected to measure SOD activity and MDA content. Measurements were obtained using commercial kits (SOD, cat. no. A001-1-1; MDA, cat. no. A003-1; Nanjing Jiancheng Biological Engineering Institute, Nanjing, China) in accordance with the manufacturer's protocol. Each experiment was repeated three times and three independent experiments were performed.

Western blotting. Myocytes were grown at 1x10⁶ cells/cm² in culture dishes as aforementioned. Following rinsing with cold D-Hanks buffer, cells were collected and lysed. Protein was extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and measured using a bicinchoninic acid (BCA) protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein (~50 μ g/lane) was separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. Membranes were then blocked with 5% fat-free milk in TBST buffer [20 mmol/l Tris-HCl (pH 7.5); 150 mmol/l NaCl and 0.05% Tween 20] and subsequently incubated with the following primary antibodies at 4°C overnight: Anti-cleaved caspase-3 (cat. no. 9661S; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Bcl2-associated X (Bax; cat. no. BS90120; Bioworld Technology, Inc., St Louis Park, MN, USA), anti-B cell lymphoma-2 (Bcl-2; cat. no. BS1511; Bioworld Technology, Inc., St Louis Park, MN, USA), anti-full length caspase-3 (cat. no. sc-56053) and polyclonal anti- β -actin (cat. no. sc-47778; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies. Each primary antibody was diluted in Tris-buffered saline with Tween 20 to 1:1,000. The mixture was washed and then incubated at room temperature for 1 h with horseradish peroxidase-conjugated immunoglobulin G secondary antibody (dilution 1:500; cat. no. 074-1506; KPL

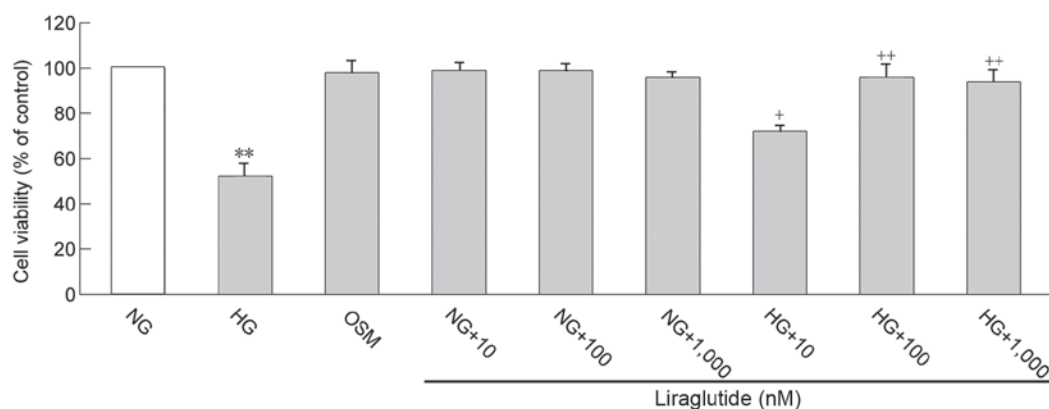


Figure 1. Cardiomyocyte viability was assessed via an MTT assay. Data are presented as the mean \pm standard deviation of three independent experiments. Cell viability was decreased in the HG group, but liraglutide treatment (10, 100 and 1,000 nmol/l) significantly improved the viability of cells exposed to HG. ** $P < 0.01$ vs. the NG group; ++ $P < 0.01$ and + $P < 0.05$ vs. the HG group. HG, high glucose; NG, normal glucose; OSM, osmotic control.

Inc., Gaithersburg, MD, USA). Membranes were developed using an ECL kit (Pierce; Thermo Fisher Scientific, Inc.) and band quantification was performed via densitometry using a gel image analysis system (GelDoc-It; UVP, LLC, Upland, CA, USA) and GeneSnap software (version 7.8; SynGene, Cambridge, UK). β -actin served as the loading control. Each experiment was repeated three times and three independent experiments were performed.

Statistical analysis. Data were presented as the mean \pm standard deviation. One-way analysis of variance was used to assess multiple differences, followed by a Tukey's post-hoc test with SPSS 19.0 software (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of HG and liraglutide on cardiomyocyte viability. Cardiomyocyte viability was assessed using an MTT assay, the results of which are presented in Fig. 1. Compared with the NG group, cell viability was significantly decreased in the HG group ($P < 0.01$). Liraglutide treatment (10, 100 and 1,000 nmol/l) significantly improved cell viability following exposure to HG ($P < 0.05$ and $P < 0.01$). However, no significant differences in cell viability were determined between the concentrations of 100 and 1,000 nmol/l liraglutide. Therefore, the current study selected 10 and 100 nmol/l liraglutide for the following experiments. All concentrations of liraglutide (10, 100 and 1,000 nmol/l) did not affect cell viability when exposed to NG, which indicates that liraglutide treatment is not cytotoxic to cardiomyocytes.

Effect of HG and liraglutide on cardiomyocyte apoptosis. The early apoptosis rate of cardiomyocytes was measured (Fig. 2). Compared with the NG group, increased early apoptosis was observed in the presence of HG ($P < 0.01$). However, liraglutide treatment (10 and 100 nmol/l) significantly suppressed the HG-induced increase of early apoptosis ($P < 0.01$). No significant difference in early apoptosis between the OSM and NG groups was identified, indicating that the effect of HG on myocyte apoptosis is independent of high osmotic pressure.

Effect of HG and liraglutide on oxidative stress. MDA content, a classic marker of oxidative damage and SOD activity, a marker of anti-oxidants, were measured. Compared with the NG group, MDA content in the HG group was significantly increased ($P < 0.01$). In contrast, SOD activity was decreased in the HG group compared with the NG group ($P < 0.01$). Treatment with liraglutide markedly decreased the HG-induced increase in MDA content and enhanced SOD activity ($P < 0.05$ and $P < 0.01$). No significant differences in MDA level and SOD activity were identified between the OSM and NG groups (Fig. 3).

Effect of HG and liraglutide on apoptosis-associated proteins. It is well known that apoptosis-associated proteins regulate the progression of apoptosis. Thus, the protein expression of Bax, Bcl-2, cleaved caspase-3 and full length caspase-3 were determined (Figs. 4 and 5, respectively). Cleaved caspase-3/full length caspase-3 was deemed to represent active caspase-3 levels. Compared with the NG group, Bax and active caspase-3 expression were significantly increased and Bcl-2 was markedly decreased in the HG group ($P < 0.01$). Following treatment with liraglutide, Bax and active caspase-3 protein levels were significantly decreased and Bcl-2 was significantly increased when compared with the HG group ($P < 0.01$). No significant differences in the OSM and NG groups were identified.

Discussion

In addition to its glucose-lowering effect, GLP-1 analogs exhibit potential clinical and cardioprotective effects. Arturi *et al* (20) revealed that treatment with liraglutide improved left ventricular function in patients with type 2 diabetes and a history of post-ischemic chronic heart failure. The Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results-A Long Term Evaluation clinical trial also demonstrated that, among patients with type 2 diabetes who were at high risk for cardiovascular events and were receiving standard therapy, those in the liraglutide group exhibited lower rates of cardiovascular events and mortality from any cause compared with those in the placebo group (11). Furthermore, Okada *et al* (21) demonstrated that treatment with liraglutide induced a reduction in reactive oxygen markers in patients with type 2 diabetes, hypothesizing

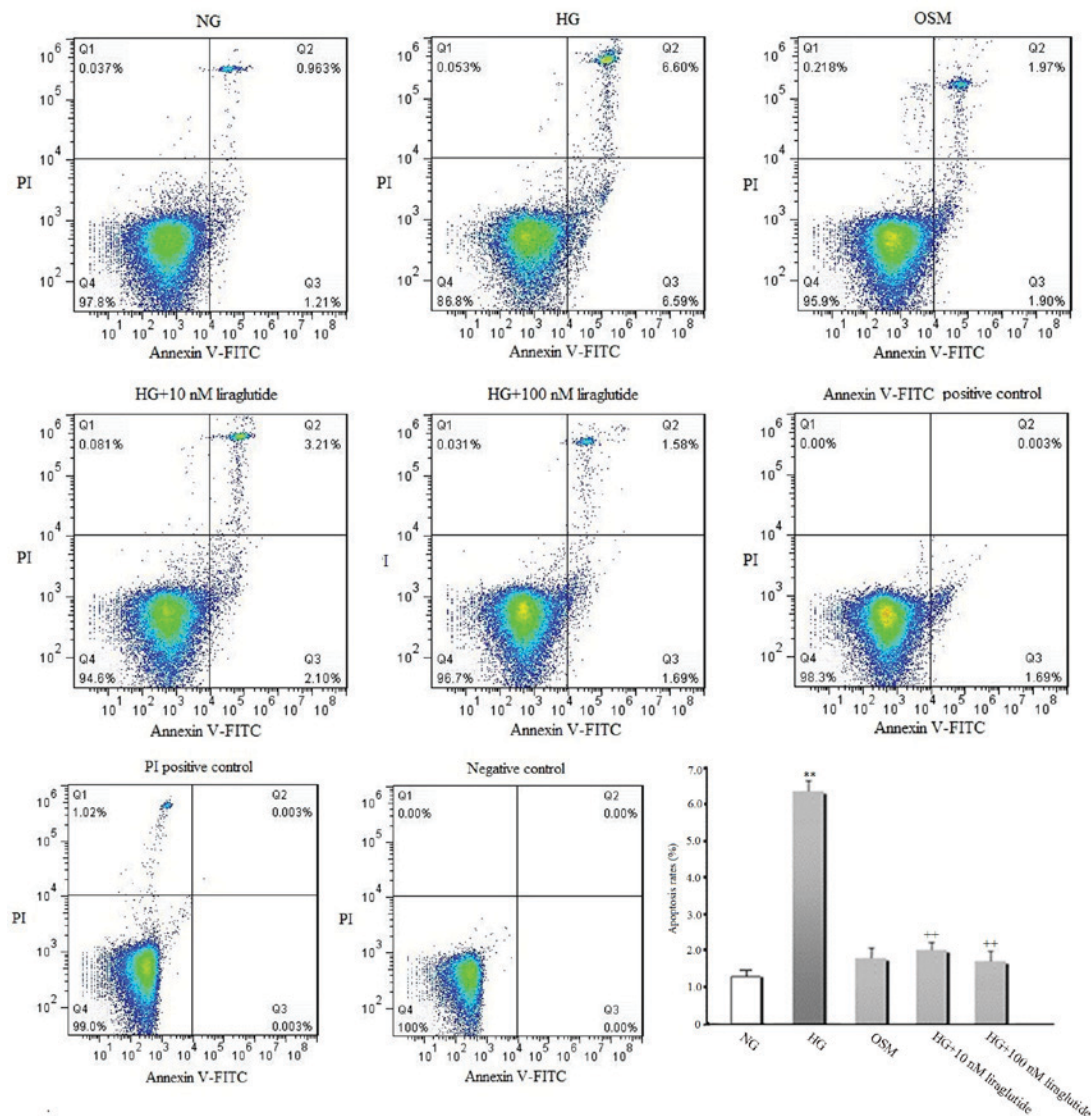


Figure 2. Percentage of early apoptotic cardiomyocytes. Data are presented as the mean \pm standard deviation of three independent experiments. Q1, necrosis cells; Q2, late apoptotic cells; Q3, viable cells; Q4, early apoptotic cells. Increased early apoptosis was identified in the presence of HG. Treatment with liraglutide significantly suppressed the HG-induced increase in early apoptosis. ** $P < 0.01$ vs. the NG group; ** $P < 0.01$ vs. the HG group. HG, high glucose; NG, normal glucose; OSM, osmotic control; PI, propidium iodide; FITC, fluorescein isothiocyanate.

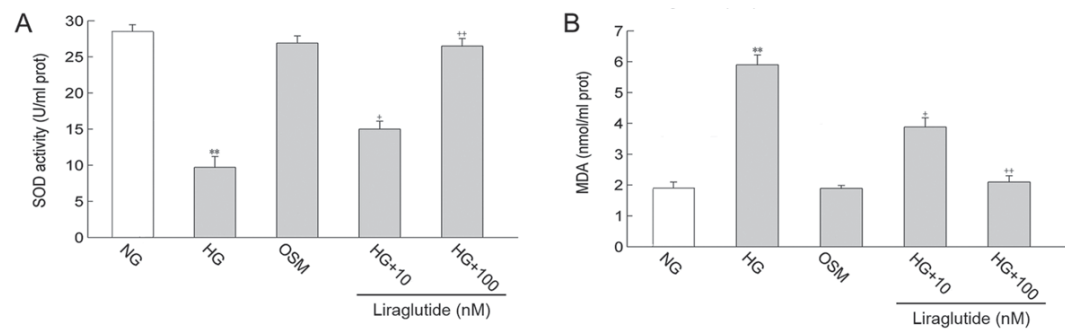


Figure 3. SOD activity and MDA content were measured in cell supernatants. Data represents the mean \pm standard deviation of three independent experiments. (A) SOD activity was decreased and (B) MDA content was significantly increased in the HG group. Treatment with liraglutide markedly decreased the HG-induced increase in MDA content and enhanced SOD activity. ** $P < 0.01$ vs. the NG group; ** $P < 0.01$ and * $P < 0.05$ vs. the HG group. SOD, superoxide dismutase; MDA, malondialdehyde; HG, high glucose; NG, normal glucose; OSM, osmotic control.

that the cardioprotective action of liraglutide may be associated with the alleviation of oxidative stress. It has also been revealed that liraglutide increases the activity of nitric oxide synthase in human endothelial cells, improving their vascular

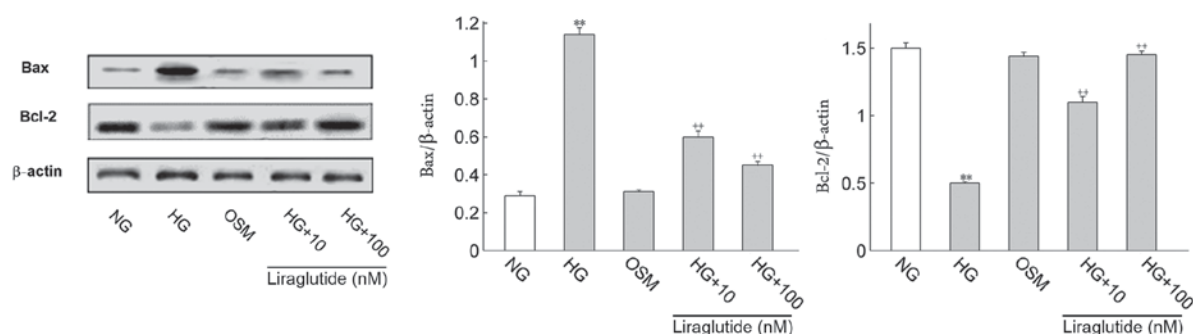


Figure 4. Bax and Bcl-2 protein expression were assessed via western blotting. Data are presented as the mean \pm standard deviation of three independent experiments. In the HG group, Bax and Bcl-2 protein levels were significantly increased and decreased, respectively. Treatment with liraglutide significantly reduced the HG-induced increase in Bax protein expression and upregulated Bcl-2 expression. ** $P < 0.01$ vs. the NG group; ++ $P < 0.01$ vs. the HG group. Bax, Bcl-2 associated X; Bcl-2, B cell lymphoma-2; HG, high glucose; NG, normal glucose; OSM, osmotic control.

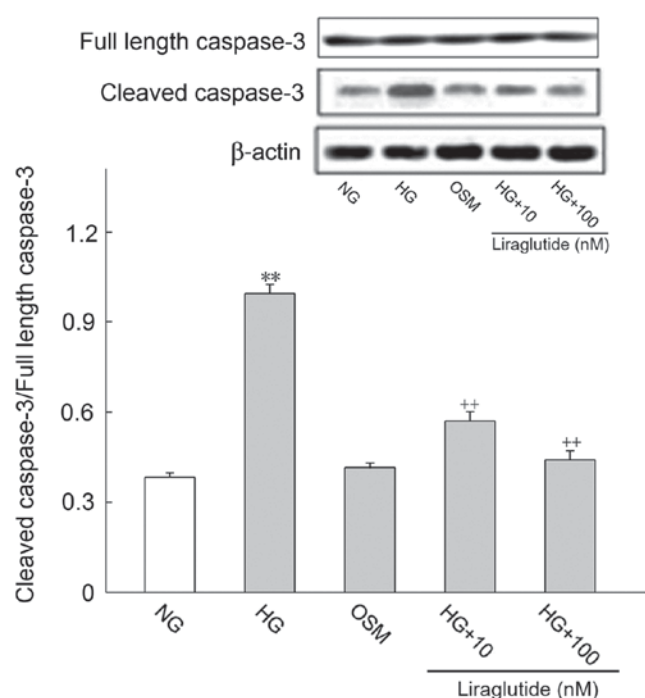


Figure 5. Cleaved caspase-3 and full length caspase-3 protein expression was determined via western blotting. Data presented as the mean \pm standard deviation of three independent experiments. In the HG group, cleaved caspase-3/full length caspase-3 protein levels were significantly increased. Treatment with liraglutide significantly reduced the HG-induced increase in cleaved caspase-3/full length caspase-3 protein expression. ** $P < 0.01$ vs. the NG group; ++ $P < 0.01$ vs. the HG group. HG, high glucose; NG, normal glucose; OSM, osmotic control.

endothelial function (22,23). These cardioprotective actions may be associated with the pleiotropic effects that liraglutide exerts on the heart.

Accumulating evidence has revealed that long-term exposure to HG results in oxidative stress and cardiomyocyte apoptosis, which serve important roles in the pathogenesis of DCM (24-26). Consistent with these observations, the results of the present study demonstrated that HG augmented oxidative stress and concurrently triggered the apoptosis signaling pathway, leading to the upregulation of the pro-apoptotic protein Bax and the downregulation of the anti-apoptotic protein Bcl-2. It has been previously reported

that the GLP-1 receptor (GLP-1R) agonist, exenatide, attenuates extracellular matrix remodeling, cardiomyocyte hypertrophy and apoptosis in experimental models of type 1 and type 2 diabetes via various mechanisms, including the suppression of oxidative stress and myocardial inflammation, as well as the regulation of endoplasmic reticulum (ER) stress and microvascular barrier function (27-29). Noyan-Ashraf *et al* (9) revealed that treatment with liraglutide reduced infarct development and improved cardiac output in murine models of type 2 diabetes with myocardial infarction (MI) compared with mice treated with metformin, and that the effects of liraglutide on enhanced survival following MI in diabetic mice were independent of glycemic control and weight loss. Their further experiment revealed that liraglutide activated cytoprotective pathways, upregulated the expression of cardioprotective genes (including protein kinase B, glycogen synthase kinase 3 β and nuclear factor erythroid factor 2-related factor 2) and inhibited the activation of caspase-3 in diabetic murine hearts, which was an effect that was superior to that of metformin (18). Additionally, Liu *et al* (16) revealed that liraglutide protects against DCM by inhibiting the ER stress pathway in rat models of type 2 diabetes and that the improvement of cardiac function by liraglutide was independent of glucose control. Inoue *et al* (17) also demonstrated that liraglutide prevents cardiac oxidative stress and apoptosis by activating the AMP-activated protein kinase (AMPK)-sirtuin 1 (Sirt1) pathway in streptozotocin-induced diabetic rats *in vivo*. These previous studies confirm that liraglutide inhibits cardiac oxidative stress and protects against DCM in diabetic animals *in vivo*. To further elucidate the protective mechanism of liraglutide against cardiomyocytes, it is necessary to perform an *in vitro* study. The present study demonstrated that liraglutide alleviates HG-induced oxidative stress and cardiomyocyte apoptosis, which may be attributable, in part, to the inhibition of Bax expression, the inhibition of caspase-3 activation and the upregulation of Bcl-2 expression. These results are congruent with those of diabetic *in vivo* models utilized in previous studies. Inoue *et al* (17) hypothesized that the beneficial effect of liraglutide on diabetic hearts may be associated with the improvement of myocardial fatty acid metabolism *in vivo* by activating the AMPK-Sirt1 pathway. The results of the current study

revealed that liraglutide exhibited a direct preventive effect on cardiomyocyte apoptosis *in vitro*. However, elucidating the mechanisms by which liraglutide exerts cardioprotection is challenging, as GLP-1R is largely expressed in atrial and not ventricular cardiomyocytes (30,31). Noyan-Ashraf *et al* (9) determined that liraglutide increased cyclic AMP formation and reduced cardiomyocyte caspase-3 activation in a GLP-1R-dependent manner. The previous study revealed that liraglutide provides cardioprotection and increased survival in GLP-1R CM^{-/-} mice, that liraglutide improved cardiac function in a GLP-1R-independent manner and that atrial GLP-1R is not required for GLP-1R agonist-mediated cardioprotection (32). Therefore, the cardioprotective effects of liraglutide may be mediated through GLP-1R-dependent and GLP-1R-independent pathways (33). Younce *et al* (34) determined that exendin-4 attenuates HG-induced cardiomyocyte apoptosis in neonatal rat ventricular myocytes *in vitro*, and that the protective effect is dependent on the inhibition of ER stress, which is downstream of oxidative stress but independent of reduced oxidative stress. However, these differences among previous studies on the cardioprotective actions of GLP-1 analogs may be associated with the different types of GLP-1 analogs used (17,34).

Clinical trials have confirmed that liraglutide exerts anti-oxidative, anti-atherosclerotic and beneficial cardiovascular effects in patients with diabetes (5-7,13-15). Consistent with these data, the present study indicated that liraglutide exerts a cardioprotective effect. The results may reveal one of the mechanisms that underlie the cardiovascular benefit of diabetic patients treated with liraglutide. The present study has certain limitations. There is an absence of data on the effect of liraglutide on myocardial apoptosis in an *in vivo* rat model of type 2 diabetes. However, a previous study has demonstrated that liraglutide inhibits cardiac myocyte apoptosis by decreasing ER stress in DCM rats (16). Furthermore, although early apoptosis rates and cell viabilities were determined via reliable methods (flow cytometry and cell viability, respectively) (35,36), terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling or DNA laddering would have provided stronger evidence to support conclusions. Additionally, the association between oxidative stress and cardiomyocyte apoptosis was not assessed in the present study. Thus, further experimental confirmation is required.

In conclusion, the current study revealed that HG augments oxidative stress and apoptosis in neonatal rat cardiomyocytes. It also demonstrated that liraglutide suppresses HG-induced oxidative stress and cardiomyocyte apoptosis, indicating that the anti-apoptotic actions of liraglutide may be, in part, due to the inhibition of Bax, the inhibition of caspase-3 activation and the upregulation of Bcl-2.

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Availability of data and materials

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

Author contributions

ZL and LC cultured cardiomyocytes and wrote the manuscript. ZQ and LN performed the superoxide dismutase and malondialdehyde measurements, and western blotting. ZH designed the current study and performed statistical analysis. All authors read and approved the final version of the manuscript.

Ethical approval and consent to participate

The animal protocol was reviewed and approved by the Laboratory Animal Ethical and Welfare Committee of Hebei Medical University (Shijiazhuang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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