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ORIGINAL

Liraglutide prevents diabetes progression in prediabetic OLETF rats

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Abstract. One of human GLP-1 analogues, liraglutide has been approved as adjuvant therapy to oral medication in T2DM. It was also shown to prevent diabetes in obese subjects and rats. However, it is unknown whether liraglutide can effectively mitigate the effects of prediabetes. We therefore investigate this by treating 12-weeks old Otsuka-Long-Evans-Tokushima fatty (OLETF) rats with liraglutide 50, 100, and 200 μg/kg, respectively twice a day for 12 weeks. Eight Long-Evans-Tokushima-Otsuka (LETO) rats with saline injection served as normal controls. Body weight, food intake, lipid profiles, inflammatory markers (fibrinogen, Hs-CRP, IL-6, TNFα, and PAI-1), glycemic metabolism and insulin sensitivity, and apoptotic factors (Bcl-2 and Bax) expression were monitored. We found that 12-week old OLETF rats had significantly increased body weight, food intake, serum levels of lipid profiles, inflammatory markers, and insulin compared to LETO rats. FPG level was significantly increased but still lower than 7mmol/L without impaired glucose tolerance (IGT). After 12 weeks, vehicle-treated OLETF rats had further deterioration in IFG, IGT, insulin resistance, lipid profiles, and inflammatory state. Pancreatic islets were hypertrophic with distorted structure, scarring, and inflammatory cell infiltration. However, in the three liraglutide-treated groups, IFG, IGT, the increased lipid profiles and inflammatory markers were reversed. Insulin resistance was similar to the level before the treatment. Moreover, liraglutide restored the islet structure, up-regulated Bcl-2 expression and down-regulated Bax expression. It indicated that liraglutide could suppress diabetes onset in OLETF rats with prediabetes, probably by reserving β cell function *via* regulating apoptotic factors as well as ameliorating lipid metabolism and inflammatory reactions.

Key words: Prediabetic, Otsuka-Long-Evans-Tokushima fatty (OLETF) rat, Inflammatory markers, Pancreatic transcription factors, Apoptotic factors

PREDIABETES is a term used to describe a state characterized by higher than normal blood glucose levels but yet to reach diabetic levels [1], which is usually related to impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG). Prediabetes has become a growing global problem. Data from USA indicate that the prevalence of IFG is about 26% and that of IGT is about 15% in the adult population based on oral glucose tolerance test (OGTT) [2]. In China, approximately 150 million Chinese adults aged 20 or older had prediabetes and the prevalence of IGT was higher than that of IFG [3]. Approximately 40% of worldwide subjects with IFG progress to diabetes over 5-10 years [4], in some newly identified IFG patients even less than 3 years [5]. Prediabetes is not only associated with diabetes but also associated with insulin resistance, increased mortality and cardiovascular risk factors such as obesity, hypertension, hyperlipidemia [6], and high in flammatory status [7]. Based on the data of the Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria in Europe

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Abbreviations: OLETF, Otsuka-Long-Evans-Tokushima fatty; LETO, Long-Evans-Tokushima-Otsuka; T2DM, type 2 diabetes mellitus; IGT, impaired glucose tolerance; IFG, impaired fasting glucose; CVD, cardiovascular disease; GLP-1, glucagon-like peptide-1; OGTT, Oral glucose tolerance test; TC, total cholesterol; TG, triglycerides; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; Hs-CRP, high-sensitivity C-reactive protein; PAI-1, plasminogen activator inhibitor-1; FINS, fasting serum concentrations of insulin; Fib, fibrinogen; IL-6, Interleukin-6; AUC, area under the curve; FPG, fasting plasma glucose; 2h PG, 2-hour postprandial glucose; ISI, insulin sensitivity index; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of β-cell function

(DECODE) study, Ning and colleagues compared cardiovascular disease mortality in individuals whose 2-hour plasma glucose in OGTT was higher than FPG with those whose 2-hour plasma glucose was equal to or lower than FPG and found that cardiovascular morbidity and mortality rose with increasing fasting glucose or 2-hour post challenge glucose levels that remained below the diagnostic cut-offs for T2DM [8]. Therefore, an early intervention during prediabetes is desirable in order to delay or prevent the onset of diabetes and reduce the mortality due to related cardiovascular disease.

The management of prediabetes includes lifestyle modification and pharmacotherapy. The beneficial effect of lifestyle modification on diabetes prevention, cardiovascular disease events, and mortality based on dietary changes and regular exercise has been reported previously [9, 10], and such effects are long-lasting [11]. However, the effective application of lifestyle modification is a challenge for many patients. Additional pharmacotherapy may be necessary, especially for individuals at high risk [9, 12]. The effect of metformin, acarbose, thiazolidinediones (TZDs), or inhibitors of the renin-angiotensin system (RAS) on prediabetes has been evaluated but it is still less effective than weight loss and physical activity [12]. Concerns also remain because many antidiabetic agents can cause weight gain, thereby exacerbating other cardiovascular risk factors. Therefore, a more effective treatment is desired.

Glucagon-like peptide 1 (GLP-1) is one of two insulinotropic hormones secreted in response to oral ingestion of glucose, indicating a promising potential therapy for diabetes. However, native GLP-1 has an exceptionally short half-life of less than 2min following administration *in vivo* due to rapid degradation by the enzyme dipeptidyl peptidase IV (DPP-IV) and rapid renal elimination [13]. Therapeutic administration of GLP-1 is thus impractical. As a result, efforts have been made for the production of GLP-1 analogues that are resistant to degradation by DPP-IV. Currently, there are two human GLP-1 analogues available as adjuvant therapy to oral medication in T2DM. One is exenatide, a short-acting human GLP-1 analogue and another is liraglutide, a long-acting human GLP-1 analogue. They can improve postprandial blood glucose by stimulating glucose dependent insulin secretion, thereby reducing the incidence of hypoglycaemia following antidiabetes treatment [14]. Moreover, they can also decrease gastric emptying and suppress appetite, thereby promoting weight loss, improve lipid profiles, and decrease systolic blood pressure [14-16]. Taking together all these benefits, human GLP-1 analogues could be excellent candidates for preventing diabetes in patients with prediabetes that is usually concomitant with obesity, hypertension, and hyperlipidemia. Rosenstock *et al.* reported that a 24-week treatment with exenatide resulted in significant weight loss and glucose tolerance improvement in patients with IFG or IGT [17]. Can liraglutide effectively help to mitigate the effects of prediabetes that exist in many patients, thereby preventing diabetes onset at a later stage? To date, there are only two clinical studies detailing its benefits in overweight subjects without prediabetes. Astrup *et al.* conducted a randomised, double-blind, placebo-controlled study on 564 overweight subjects and showed that 20-week treatment with one of four doses of liraglutide significantly reduced body weight, blood pressure and the prevalence of prediabetes compared with placebo or orlistat [18], and these benefits were sustained with a 2-year treatment [19]. The transition from obesity to prediabetes is a long-term progression. Further studies in prediabetic status are necessary. Recently, Cummings *et al.* [20] reported that liraglutide treatment delayed diabetes onset in prediabetic UCD-T2DM rats by reducing energy intake and body weight, and by improving insulin sensitivity, improving lipid profiles, and maintaining islet morphology. However, they started to treat animals from 8 weeks of age with normal FPG and non-FPG levels, which is far earlier than the prediabetic stage. Whether liraglutide can reverse prediabetes and its associated risk factors such as inflammatory status and hyperlipidemia still remains obscured. We therefore conducted the present study to investigate the effect and underlying mechanisms of liraglutide on prediabetes and its related pathological conditions in OLETF rats.

Materials and Methods

1. Animals and composition of experimental groups

All animal experimental procedures were carried out in accordance with the principles of laboratory animal care and approved by the ethical committee in the Southern Medical University (Guangzhou, China). Four-week-old male OLETF rats and age-matched non-diabetic control male LETO rats were generously provided by the Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). All rats were kept individually in polycarbonate cages with free access to standard rodent chow and tap water in

a temperature-controlled environment (21±2°C, lights on 7:00 a.m. to 7:00 p.m.). We used 12 weeks old male OLETF rats as a prediabetic model and treated them with three doses of liraglutide (50, 100, or 200 μg/kg, Novo Nordisk Pharmaceuticals Co., Ltd) or 0.9% saline intraperitoneally, twice daily, 8 rats in each group. In the meantime, eight LETO rats served as normal controls with saline treatment.

2. Experimental protocols

Body weight and food intake were monitored at the same time once a week. Blood samples were taken from the tail vein for overnight fasting blood glucose assessment at 0, 2, 4, 6, 8, 10, and 12 weeks after drug intervention using an OneTouch UltraVue glucose meter (LifeScan, Inc., Milpitas, CA). The blood samples at 0 and 12 weeks were also used for the measurement of lipid profile, including total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-c), and HDL-c with an automatic biochemical analyzer (Aeroset, American), and inflammatory markers, including serum high-sensitivity C-reactive protein (Hs-CRP), interleukin-6 (IL-6), fibrinogen (Fib), and plasminogen activator inhibitor-1 (PAI-1) with a mouse enzyme-linked immunosorbent assay (ELISA) (Uscnlife, Wuhan EIAab Science Co., Ltd, Wuhan, China). The fasting serum insulin (FINS) level and serum insulin level at OGTT 30min (INS 30min) were measured by ELISA at 0 and 12 weeks. The indices for insulin sensitivity and beta cell function before and after treatment were calculated subsequently, including insulin sensitivity index (ISI) = 1 / FPG x FINS, the homeostasis model assessment of insulin resistance (HOMA-IR) = (FPG x FINS)/ 22.5 [21, 22], the homeostasis model assessment of ß-cell function HOMA-β=20×FIns/(FBG-3·5), and the ratio of insulin incremental value to glucose incremental value at 30 min after the meal ΔIns30/ΔGlu30 = (Ins30-Ins0)/(Glu30-Glu0).

Oral glucose tolerance test (OGTT) was performed in all rats at 0, 2, 4, 6, 8, 10, and 12 weeks of experiment. Briefly, a 50% glucose solution (2 g/kg body weight) was orally administrated with an 18-gauge gavage needle after overnight fast, and then blood samples were collected from the tail vein in a conscious state at 0, 30, 60, and 120 minutes following the glucose challenge to determine plasma glucose levels. Plasma glucose AUC (PG-AUC) during OGTT in each group was derived according to the trapezoidal rule [23] and the differences between groups were compared.

There is not any standard available for diagnosing diabetes and prediabetes in the rat model. Therefore, we follow others [20, 24] to use WHO criteria to diagnose diabetes and prediabetes. Diabetes is defined as a FPG > 7.0 mmol/L or a 2 h PG > 11.1 mmol/L during an OGTT. IFG is defined by a FPG > 5.6 mmol/L but < 7.0 mmol/L. IGT is defined by a FPG < 7.0 mmol/L with a 2 h PG during an OGTT > 7.8 mmol/L and < 11.1 mmol/L. Pre-diabetes is isolated IFG or isolated IGT or combined. Therefore, we used the same diagnosis criteria for the present study.

At the end of 12 weeks of treatment, all rats were euthanized by administration of pentobarbital (50 mg/kg). The pancreatic tissues were rapidly removed, fixed immediately in 4% paraformaldehyde solution for 24 hours and then paraffin-embedded, thin-sectioned (3-5 μm) for routine histopathological analysis and for immunohistochemical analysis.

3. Histopathology and immunohistochemistry

Haematoxylin & Eosin stain was performed following standard protocols and then morphological changes were observed under a light microscope (BX41TF, OLYMPUS). The pancreatic islets were sampled from all pancreatic regions to avoid bias in quantification and at least 25 islets were analyzed per mouse. All islets were circled to calculate the size.

Immunohistochemical assay was performed to detect the protein expression of antiapoptotic Bcl-2 and proapoptotic Bax in pancreatic islets. Briefly, paraffin-sections were rehydrated in a descending xylene/ethanol series and endogenous peroxidase activity was quenched with methanol containing 3 % hydrogen peroxide for 10 min at room temperature. To avoid non-specific reactions with the background, the sections were incubated with normal goat serum at 37°C for 20 minutes prior to incubation with specific antibodies against Bcl-2, and Bax (Santa Cruz Biotechnology, USA) at 4 °C overnight in a humidified chamber. After rinsing in PBS buffer (0.01M, pH 7.4) three times, they were incubated with secondary antibodies (mouse anti-rabbit IgG, dilution, 1:200; DAKO, Denmark) at 37°C for 20 minutes. Sections were then washed in PBS buffer and finally incubated with DAB (3,3′-diaminobenzidine tetrahydrochloride) containing 0.01% H_2O_2 in Tris-HCl buffer (0.05 M, pH 7.6), dehydrated, and mounted. After staining, the sections were observed under a light microscope. To quantify staining for BCL-2 and Bax protein, integrated optical density (IOD) was calculated

as the product of staining area and intensity and presented as IOD/μm^2.

4. Statistical analysis

SPSS 16.0 software was used for statistical analysis. Data throughout were stated as means ± SEM unless otherwise specified. General effects were tested using one way ANOVA followed by Bonferroni or Dunnett's test for individual comparisons of means (because 3 rats died accidentally, 2 in the liraglutide 100 μg/kg group and 1 in the liraglutide 200 μg/kg group before the end of experiment). Differences between measurements before and after treatment were analysed by use of Wilcoxon signed rank tests. A two-tailed Pearson test was performed for correlation analysis between the variables. $p < 0.05$ was considered statistically significant.

Results

1. The effect of liraglutide on food intake and body weight

Food intake in OLETF rats was significantly more than that of LETO rats before liraglutide treatment ($p < 0.0001$). It was markedly suppressed by liraglutide treatment in the first week (all $p<0.05$) in a dose-independent manner, and then gradually increased to a similar level seen in vehicle-treated OLETF rats, which was then maintained at the same level throughout the remainder of the experimental period. After 12-week treatment, the amounts of food intake in the three liraglutide groups were similar (50 μg/kg group, 34.73 ± 0.49 g/day; 100 μg/kg group, 32.59 ± 0.57 g/day; 200 μg/kg group, 33.17 ± 0.53 g/day) and significantly more than that of LETO rats (25.00 ± 0.41 g/day) (all $p<0.0001$). They were slightly reduced but not statistically significant from that of vehicle-treated OLETF rats (37.41 ± 0.49 g/day) (all p =NS) (Fig. 1A).

Similar to food intake, body weight in OLETF rats was significantly greater than that of LETO rats before treatment (416.8±5.8g *vs*. 325.8±5.6g, $p<0.0001$), which was significantly reduced with liraglutide treatment in a dose-independent manner within the first week compared with that of vehicle-treated OLETF rats (all $p<0.05$). This then gradually increased from the second week parallel to vehicle-treated animals and normal controls, which were significantly greater than LETO rats (all $p<0.01$) but still less than that of vehicle-treated OLETF rats (all $p<0.05$) despite similar levels of food intake (Fig. 1B). These results suggested that liraglutide only had an acute effect on food intake but its beneficial effect on weight loss was sustained and independent of food intake.

2. The effect of liraglutide on glycemic metabolism and insulin secretion and sensitivity

As shown in Fig. 2, FPG levels in vehicle-treated OLETF rats were significantly higher than in LETO rats ($p<0.05$) but only reached diabetic levels at the age of 24 weeks. Similarly, 2-hour postprandial blood glucose level (2h-PG) was normal until the age of 24 weeks, suggesting that 12 weeks old OLETF rats had IFG but not IGT. In addition, FPG was independent of food intake and bodyweight gain in vehicle-treated OLETF rats (p=NS) (Fig. 3) but 2h-PG was positively correlated to bodyweight gain (R=0.762, p=0.028). After one week of treatment, the elevation in FPG, 2h-PG and the glucose area under the curve ($AUC_{0\text{-}120}$ min) was significantly restrained by liraglutide in a dose-independent manner (all $p<0.05$ compared with that of vehicle-treated OLETF rats) and maintained at the same level as LETO rats throughout the rest of the experiment period.

Before treatment, 12-week old OLETF rats had significantly increased FINS, FIN 30min, and HOMA-IR levels and decreased ISIx1000, HOMA-β and ΔIns30/ΔGlu30 levels compared with LETO rats (all $p<0.05$), suggesting insulin resistance and impaired beta cell function. After 12-week treatment, vehicle-treated OLETF rats had further increased FINS and HOMA-IR levels and decreased ISIx1000, HOMA-β and ΔIns30/ΔGlu30 levels (all $p<0.05$). The above abnormalities were ameliorated by liraglutide treatment (Table 1). Although FINS was maintained at a similar level before treatment, HOMA-β and ΔIns30/ΔGlu30 levels were significantly improved with liraglutide 200μg/kg twice daily (all $p<0.05$).

By the end of the 12-week intervention period, 7 of 8 (87.5%) vehicle-treated OLETF rats progressed to diabetes characterized by significantly increased FPG and 2h-PG levels, insulin resistance and impaired beta cell function. However, FPG levels were reversed to normal in 9 of 21 (42.9%) liraglutide-treated OLETF rats whilst none of the liraglutide-treated OLETF rats progressed to diabetes ($p<0.0001$ compared with vehicle-treated animals).

3. The effect of liraglutide on lipid profiles and inflammatory state

Compared with LETO rats, serum level of TC was significantly increased in OLETF rats from the age of

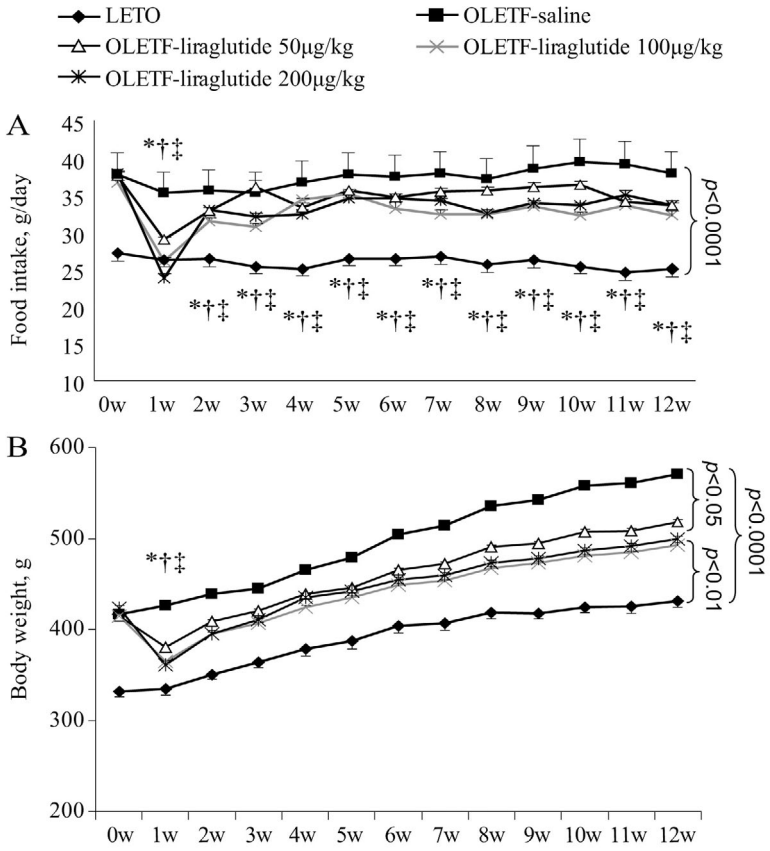

Fig. 1 The changes of food intake with treatment
A: The food intake in liraglutide-treated OLETF rats was significantly reduced after first week and then increased after the second week. p=NS when comparing liraglutide-treated groups with saline controls at the end of 12 weeks of treatment. B: Body weight in liraglutide-treated OLETF rats was significantly reduced during the first week and then increased from the second week but remained significantly lower than saline controls at the end of 12 weeks of treatment. Values are means ± SEM of 6-8 rats. *p<0.05 compared with OLETF-liraglutide 50μg/kg group; †p<0.05 with OLETF-liraglutide 100μg/kg group; ‡p<0.05 with OLETF-liraglutide 200μg/kg group

12 weeks to 24 weeks (all p<0.05). Serum level of TG was also significantly increased at the age of 24 weeks (p<0.05). After 12-week treatment with liraglutide, serum level of TC but not TG was significantly reduced (p<0.05 compared with that before treatment). There was no difference in terms of HDL and LDL between groups or before and after liraglutide treatment (Table 2).

The changes in inflammatory markers are shown in Table 3. The serum levels of fibrinogen, Hs-CRP, IL-6, TNF-α and PAI-1 were significantly higher in OLETF rats compared with LETO rats at the age of 12 weeks (all p<0.05). PAI-1 and fibrinogen levels were further increased in vehicle-treated OLETF rats at the age of 24 weeks (all p<0.05). After 12 weeks of treatment, IL-6 and TNF-α levels were significantly reduced by all doses of liraglutide, while Hs-CRP level was only reduced by liraglutide 200 μg/kg and PAI-1 level by liraglutide 50 μg/kg (all p<0.05 compared with the levels before treatment).

Moreover, a positive correlation was found between the bodyweight gain and serum level of Hs-CRP (R=0.714, p=0.047), and between the serum level of TG and PAI-1(R=0.881, p=0.004) in 24 weeks old vehicle-treated OLETF rats. Such relationships did not appear in the liraglutide-treated animals.

4. The effect of liraglutide on pancreas morphology

Of the three groups of liraglutide-treated rats, the histology and immunohostochemistry studies were only performed in the liraglutide 100μg/kg group because the dose of liraglutide 100μg/kg twice daily in rat can be converted to human equivalent dose of 0.032mg/kg (1.92mg/day assuming 60kg human) according to FDA's guidance [25], which is similar to the dose com-

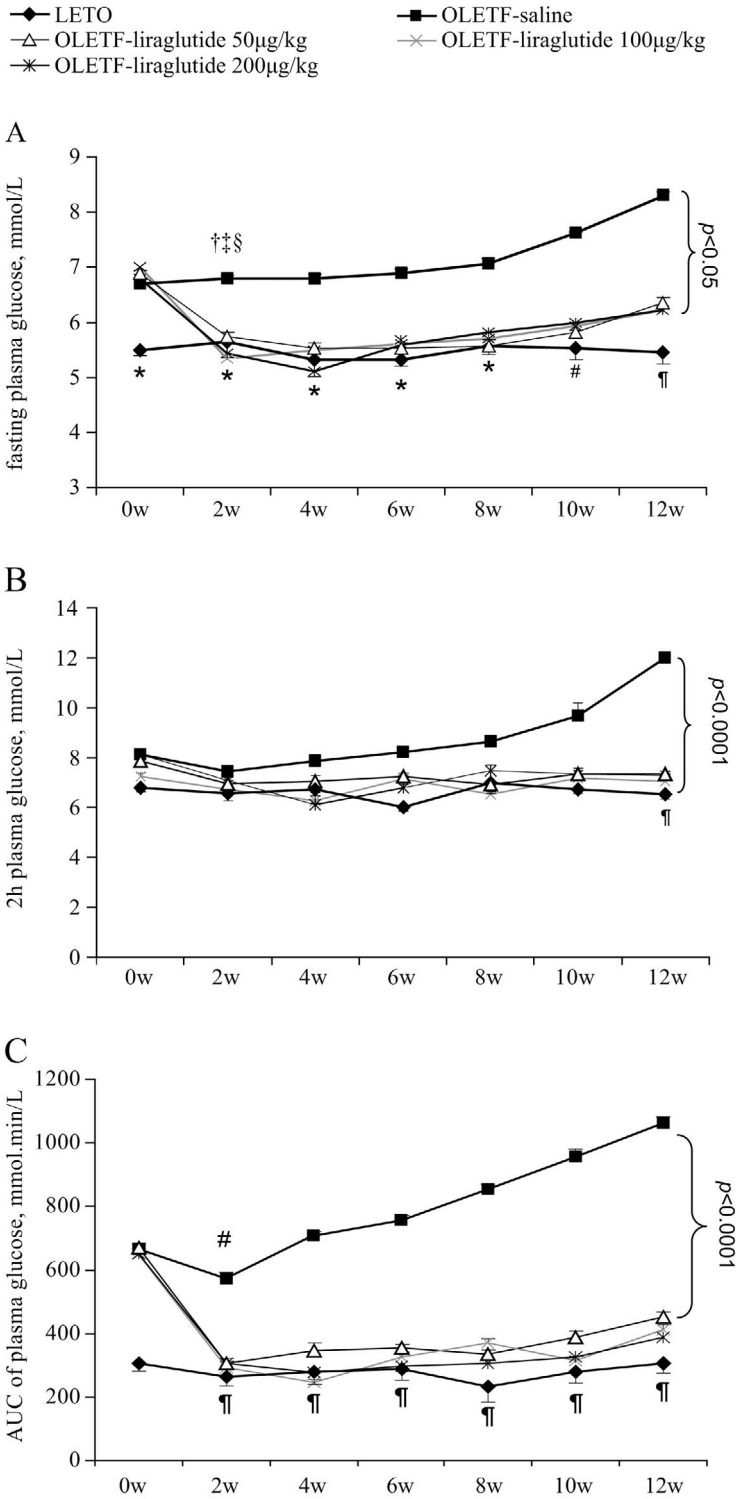

Fig. 2 The effect of liraglutide on fasting plasma glucose (FPG) and glucose tolerance
Effect of liraglutide on fasting plasma glucose (A), 2h postprandial glucose (B) and the area under the plasma glucose concentration-time curve (C) during the OGTT test. Values are means ± SEM of 6-8 rats. * $p<0.05$ LETO *vs*. OLETF saline; # $p<0.001$ LETO *vs*. OLETF saline; ¶ $p<0.0001$ LETO *vs*. OLETF saline; † $p<0.05$ OLETF saline group *vs*. liraglutide 50μg/kg; ‡ $p<0.05$ OLETF saline group *vs*. liraglutide 100μg/kg; § OLETF saline group *vs*. liraglutide 200μg/kg; # $p<0.001$ OLETF saline group *vs*. each liraglutide-treated group

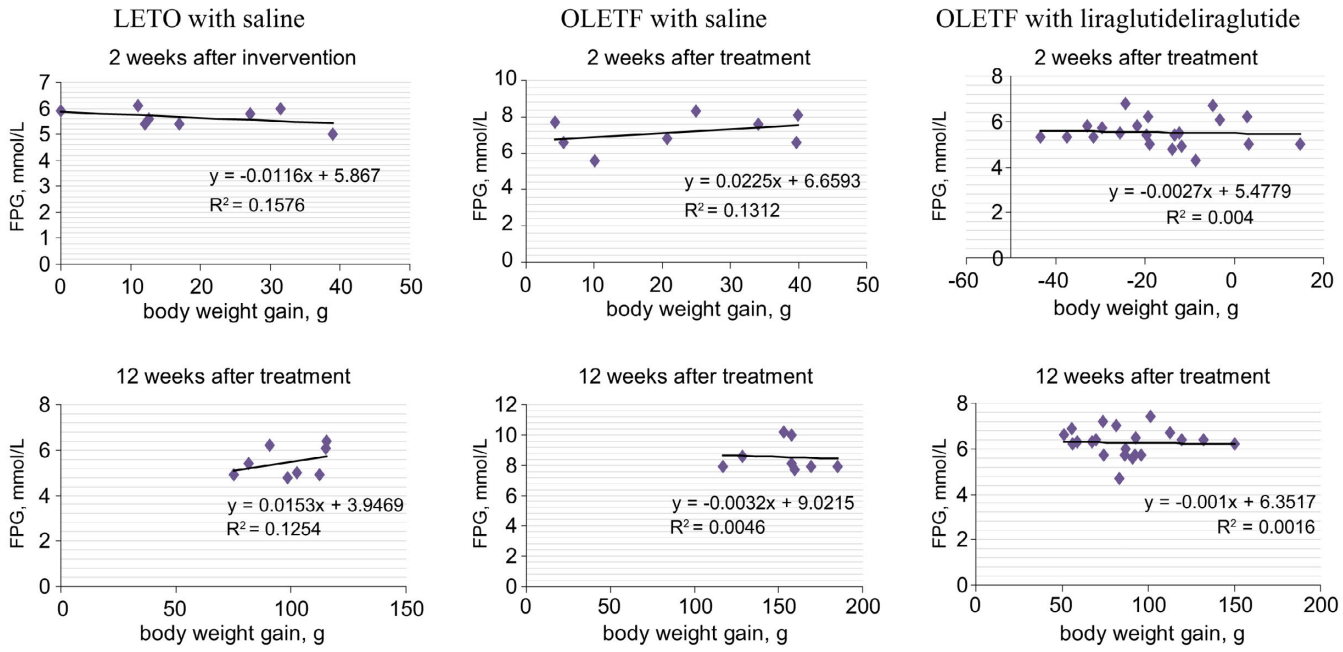

Fig. 3 The relationship between body weight gain and fasting plasma glucose (FPG)
FPG remained at similar level whilst body weight gain was different in both LETO and OLETF rats.

Table 1 Insulin secretion, sensitivity and beta cell function after 12-weeks intervention

	LETO rats	IGT - OLETF rats			
	Saline	Saline	Liraglutide		
	(N=8)	(N=8)	50 μg/kg (N=8)	100 μg/kg (N=6)	200 μg/kg (N=7)
FINS (μIU/mL)					
Before therapy	27.21±0.36	37.49±0.78*	37.76±1.08*	37.32±1.25*	37.59±1.21*
After therapy	27.46±0.60	51.03±1.12*	39.86±0.94*#	38.19±1.00*#	37.03±1.21#
	p=NS	*p*=0.000	*p*=NS	*p*=NS	*p*=NS
INS 30min (μIU/mL)					
Before therapy	78.84±1.10	94.22±1.25*	93.42±1.81*	93.08±2.60*	92.91±2.74*
After therapy	73.10±1.48	99.51±2.18*	119.57±2.49*	122.20±2.54*	125.91±3.75*
	p=NS	*p*=NS	*p*=0.001	*p*=0.004	*p*=0.009
ISI×1000					
Before therapy	7.06±0.18	4.12±0.12*	4.04±0.08*	4.12±0.12*	4.07±0.12*
After therapy	6.29±0.60	2.37±0.09*	4.06±0.11*#	4.34±0.11*#	4.73±0.20#
	p=NS	*p*=0.000	*p*=NS	*p*=NS	*p*=NS
HOMA-IR					
Before therapy	6.32±0.17	10.83±0.31*	11.03±0.21*	10.83±0.33*	10.97±0.34*
After therapy	7.28±0.48	19.55±0.87*	11.27±0.36*#	10.54±0.28#	10.40±0.48#
	p=NS	*p*=0.001	*p*=NS	*p*=NS	*p*=NS
HOMA-β					
Before therapy	323.46±20.63	251.92±9.82*	248.03±14.43*	247.99±14.60*	247.41±14.87*
After therapy	331.16±3.19	205.75±4.20*	290.99±12.79#	288.62±11.23#	294.41±14.24#
	p=NS	*p*=0.010	*p*=NS	*p*=NS	*p*=0.049
ΔIns30/ΔGlu30					
Before therapy	11.23±0.85	5.21±0.40*	5.48±0.30*	5.08±0.29*	5.21±0.20*
After therapy	11.99±0.31	3.85±0.19*	9.00±0.35#	8.90±0.37#	9.55±0.25#
	p=NS	*p*=0.049	*p*=0.005	*p*=0.016	*p*=0.001

Data are means ± SEM, unless otherwise noted. FINS, fasting serum insulin; INS 30min, serum insulin 30min after glucose challenge; ISI, insulin sensitivity index; HOMA-IR, homeostasis model of insulin resistance; HOMA-β, homeostasis model assessment for *β*-cell function; ΔIns30/ΔGlu30, the ratio of the change in insulin to glucose response over the first 30 min of the OGTT; * $p<0.05$ compared with the LETO-saline group; # $p<0.05$ compared with the IGT-OLETF saline group; NS, not statistically significant

Table 2 The changes of lipid profiles with liraglutide treatment (means ± SEM)

	LETO rats	IGT - OLETF rats			
	Saline	Saline	Liraglutide		
	(N=8)	(N=8)	50 μg/kg (N=8)	100 μg/kg (N=6)	200 μg/kg (N=7)
TC (mmol/L)					
Before therapy	2.59±0.05	3.01±0.05*	2.84±0.04	3.05±0.12*	2.94±0.02
After therapy	2.02±0.07	2.56±0.08*	2.21±0.02	2.24±0.05	2.13±0.02#
	p=NS	*p*=0.039	*p*=0.001	*p*=0.002	*p*=0.001
TG (mmol/L)					
Before therapy	0.40±0.01	0.64±0.06	0.55±0.04	0.70±0.10	0.64±0.02
After therapy	0.36±0.04	1.09±0.06*	0.71±0.04	0.40±0.13#	0.51±0.03#
	p=NS	*p*=0.020	*p*=NS	*p*=NS	*p*=NS
HDL (mmol/L)					
Before therapy	1.75±0.04	2.0±0.06	1.68±0.16	2.06±0.08	1.97±0.07
After therapy	1.22±0.06	1.57±0.09	1.36±0.06	1.39±0.03	1.33±0.04
	p=NS	*p*=NS	*p*=NS	*p*=NS	*p*=NS
LDL (mmol/L)					
Before therapy	0.17±0.00	0.18±0.00	0.17±0.00	0.17±0.01	0.16±0.00
After therapy	0.33±0.03	0.27±0.00	0.19±0.02	0.30±0.07	0.26±0.01
	p=NS	*p*=NS	*p*=NS	*p*=NS	*p*=NS

Fib, fibrinogen; Hs-CRP, high-sensitivity C-reactive protein; IL-6, Interleukin-6; PAI-1, plasminogen activator inhibitor-1; TC, total serum cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; *$p<0.05$ compared with the LETO-saline group; #$p<0.05$ compared with the IGT-OLETF saline group; NS, not statistically significant

Table 3 The changes of inflammatory markers with liraglutide treatment (means ± SEM)

	LETO rats	IGT - OLETF rats			
	Saline	Saline	Liraglutide		
	(N=8)	(N=8)	50 μg/kg (N=8)	100 μg/kg (N=6)	200 μg/kg (N=7)
Fib (U/mL)					
Before therapy	55.38±1.92	74.04±1.66*	76.03±1.43*	73.66±4.01*	77.03±1.14*
After therapy	62.51±3.64	94.20±1.32*	80.45±1.21*	81.60±3.40*	78.15±1.88
	p=NS	*p*=0.003	*p*=NS	*p*=NS	*p*=NS
Hs-CRP (ng/mL)					
Before therapy	3.56±0.16	5.53±0.17*	5.37±0.14*	5.32±0.41*	5.39±0.07*
After therapy	4.22±0.35	5.72±0.18*	4.14±0.17	3.90±0.48#	3.68±0.12#
	p=NS	*p*=NS	*p*=NS	*p*=NS	*p*=0.006
IL-6 (pg/mL)					
Before therapy	5.11±0.38	8.89±0.27*	8.18±0.20*	8.18±0.56*	8.30±0.22*
After therapy	4.73±0.38	8.72±0.19*	5.63±0.32#	4.79±0.90#	5.11±0.26#
	p=NS	*p*=NS	*p*=0.022	*p*=0.023	*p*=0.023
TNF-α (ng/ml)					
Before therapy	31.70±3.00	48.90±2.62*	47.00±2.30*	47.47±3.24*	48.81±4.20*
After therapy	34.55±3.62	53.22±2.09*	38.21± 1.19#	31.62±6.30#	31.98± 5.02#
	p=NS	*p*=NS	*p*=0.047	*p*=0.023	*p*=0.022
PAI-1 (ng/mL)					
Before therapy	26.45±1.54	48.67±3.01*	50.42±2.04*	45.86±5.71*	54.98±1.81*
After therapy	22.03±2.73	63.37±0.99*	27.74±3.19#	37.34±21.88#	34.07±1.66#
	p=NS	*p*=0.026	*p*=0.004	*p*=NS	*p*=NS

Fib, fibrinogen; Hs-CRP, high-sensitivity C-reactive protein; IL-6, Interleukin-6; PAI-1, plasminogen activator inhibitor-1; *$p<0.05$ compared with the LETO-saline group; #$p<0.05$ compared with the IGT-OLETF saline group; NS, not statistically significant

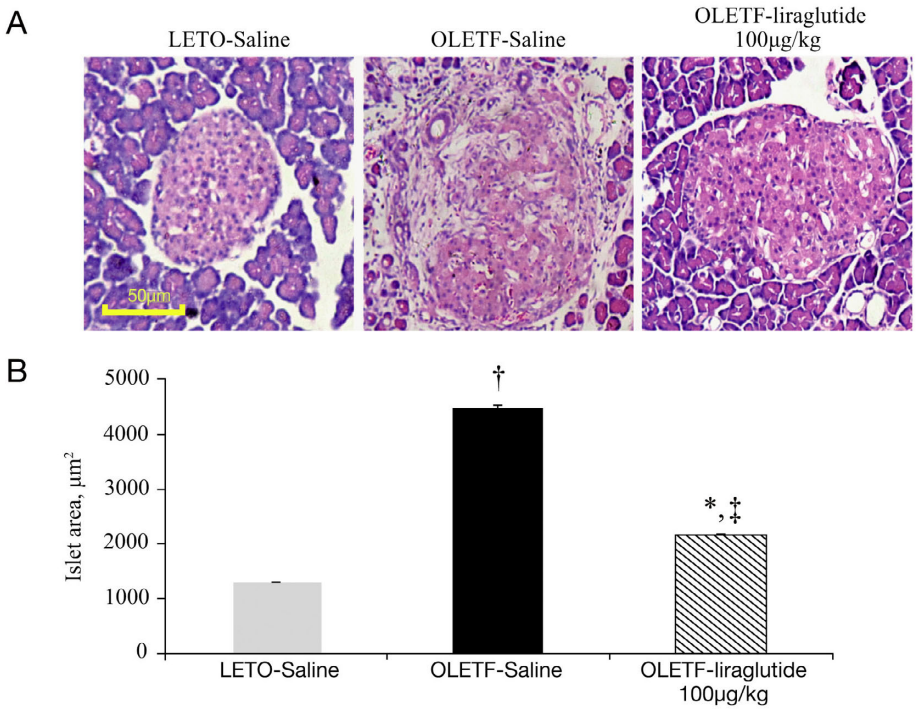

Fig. 4 Pancreatic islets histology
A, Haematoxylin & Eosin staining of representative pancreatic islets. Compared with normal structure of pancreatic islets in LETO rats, pancreatic islets in OLETF rats treated with saline were significantly enlarged and the boundaries became irregular and disappeared. A distorted structure and severe scarring of the stroma were observed. After 12 weeks of treatment with liraglutide, the structure of pancreatic islets was restored and the size of islets was reduced. B, the average area of pancreatic islets in LETO saline controls, OLETF saline-treated and OLETF liraglutide-treated rats. Scale bar = 50μm; N=7-8; † $p<0.01$ comparing OLETF saline group and LETO saline group; *$p<0.05$ comparing OLETF liraglutide 200μg/kg group with LETO saline group; ‡ $p<0.05$ comparing OLETF liraglutide 200μg/kg group with OLETF saline group

monly applied clinically.

At the age of 24 weeks, the LETO group showed typical pancreas morphology, with normal structure of exocrine acini and pancreatic islets. However, in vehicle-treated OLETF rats, a distorted structure, hypertrophic islets, severe scarring of the stroma, and a large amount of inflammatory cell infiltratation were observed. The islet boundaries became irregular and disappeared. After 12-week treatment with liraglutide, the structure of pancreatic islets was restored and the size of islets was reduced (Fig. 4A) as indicated by significantly reduced islet area ($p<0.05$ compared with vehicle-treated OLETF rats) though it was still larger than vehicle-treated LETO rats ($p<0.05$) (Fig. 4B). There was no difference regarding the number of islets among three groups.

5. The effect of liraglutide on apoptotic factors

Immunohistochemistry staining detected a decreased protein expression of anti-apoptotic factor Bcl-2 and increased protein expression of pro-apoptotic factor Bax in pancreatic islets of OLETF rats at the age of 24 weeks compared with LETO controls (Fig. 5). However, such changes were significantly reversed by 12-week treatment with liraglutide 100μg/kg twice daily as indicated by significantly increased Bcl-2 IOD and decreased Bax IOD though they were still significantly different from that in the LETO controls (all $p<0.05$ comparing three groups) (Table 4). These results suggested that liraglutide can down-regulate pro-apoptotic factor Bax and upregulate anti-apoptotic factor Bcl-2, which may contribute to the improvement of pancreatic islet structure and function.

Discussion

The present study has several main findings. 1) OLETF rats had IFG, obesity, high cholesterol, high inflammatory state, hyperinsulinemia, and insulin resistance at the age of 12 weeks and progressed to diabetes with hyperinsulinemia, hyperglycemia,

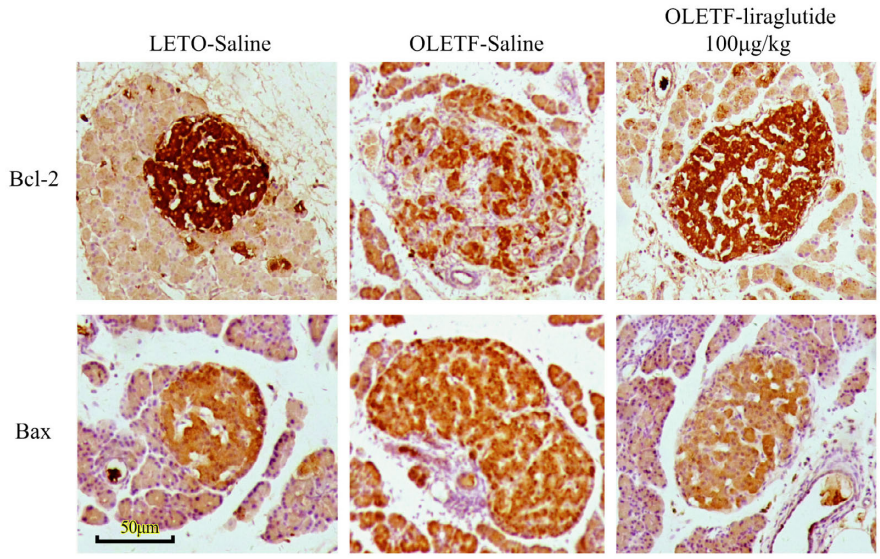

Fig. 5 The protein expression of Bcl-2 and Bax in pancreatic islets by immunohistochemistry
At the age of 24 weeks, OLETF rats had a decreased Bcl-2 protein expression and increased Bax protein expression compared with LETO rats. However, such changes were reversed by liraglutide 200μg/kg treatment. Scale bar = 50μm

Table 4 The effect of 12-week treatment with liraglutide on the BCL-2 and Bax protein expression in pancreatic islets detected by immunohistochemistry. Data expressed as mean ± SEM, IOD/μm^2

	LETO-Saline (N=8)	OLETF-Saline (N=8)	OLETF-liraglutide 200μg/kg (N=7)
BCL-2	0.3196±0.01	0.0317±0.02*	0.1214±0.01†‡
BAX	0.0938±0.01	0.1631±0.01*	0.1146±0.02†‡

* $p<0.001$ compared with the LETO-Saline group; † $p<0.05$ compared with the LETO-Saline group; ‡ $p<0.05$ compared with the OLETF-Saline group

hypertriglycemia, insulin resistance, and impaired beta cell function at the age of 24 weeks. Our findings surrounding the diabetic phenotype of OLETF rats are similar to Kawano's report [26]. 2) Liraglutide treatment only acutely reduced food intake within the first week. Bodyweight loss was concomitantly induced by liraglutide in the first week but the effect was consistent until the end of treatment and independent of food intake. 3) Three doses of Liraglutide treatment suppressed IFG, IGT and insulin resistance. It also improved hypertriglycemia and inflammatory state. 4) Liraglutide treatment preserved islet morphology. 5) Liraglutide up-regulated antiapoptotic Bcl-2 and down-regulated proapoptotic Bax expression in islets, which may contribute to its protective effect on islet structure and function.

Since it was developed in 1991, OLETF rat model has contributed substantially to understanding the pathophysiology and treatment of T2DM and its complications [27] because it spontaneously develops obesity, hyperlipideamia, glucose intolerance after 18 weeks of age, and late onset of diabetes at 23 weeks of age [26, 28]. In addition, a gene mutation similar to OLETF rats, i.e., cholecystokinin-1 (CCK-1) and cholecystokinin-2 (CCK-2) receptor polymorphism has been found in patients with obesity and T2DM [6], and a genetic locus related to CCK has also been identified in Mexican Americans, which influences BMI and progresses to T2D [29]. Therefore, OLETF rats younger than 18 weeks could be an ideal physiological model for studying the treatment of prediabetes [30]. In fact, we found obesity, hyperlipideamia, glucose intolerance and insulin resistance have already occurred in 12 weeks old OLETF rats.

As mentioned previously, human GLP-1 analogues can decrease gastric emptying and suppress appetite, thereby promoting weight loss. Our results have clearly shown that the effect of liraglutide on food intake was acute and short term but its effect on weight loss was sustained and independent of food intake, suggesting that the effect of liraglutide on

weight loss may be due to other mechanisms rather than decreased food intake. Similar findings were reported with exenatide treatment. Mack *et al.* [31] used exenatide to treat high-fat-fed rats and found that the food intake was significantly reduced during the first week of exenatide treatment. But during weeks 3 and 4, exenatide-treated rats displayed food intake levels similar to vehicle-treated rats while the bodyweight loss in these animals was sustained. In addition, the weight loss was accompanied by a loss of fat tissue, with a sparing of lean mass. They supposed that the underlying mechanism of bodyweight loss by exenatide was satiety-related rather than altering locomotion. In the UCD-T2DM rat model of diabetes, Cummings *et al.* [20] reported a sustained reduction of energy intake by liraglutide treatment. But they also found that liraglutide-treated animals had a lower percentage of body fat mass and better plasma glucose control compared with food-restricted animals despite similar weight loss, suggesting a preferential increase of lipid oxidation with liraglutide treatment rather than reduced energy intake. Although we did not measure body fat mass in the present study, the reduction of TC and TG in liraglutide-treated animals also indicated an increase in lipid oxidation due to liraglutide treatment. In our prediabetic OLETF rats, the effect of liraglutide on weight loss may be suppressed by decreased food intake due to the lack of CCK-A receptors, which results in a reduced ability to process nutrient elicited gastrointestinal satiety signals in this rat model. Nevertheless, our results and others' findings further confirm that the effect of GLP-1 agonists on weight loss is strong and beneficial regardless of changes in food or energy intake.

In the present study, liraglutide effectively suppressed the diabetes onset and reversed IFG to normal in nearly 50% of OLETF rats. The transition from pre-diabetes to diabetes involves at least two major underlying mechanisms, namely, insulin resistance and impaired beta cell function. Our data suggest that both abnormalities have initiated in 12 weeks old OLETF rats and further deteriorate at the age of 24 weeks. Liraglutide retards the deterioration of insulin resistance but significantly improves IPG and impaired beta cell function. The hyperinsulinemia and insulin resistance in some liraglutide-treated OLETF rats may be related to remaining obesity though liraglutide did reduce their bodyweight to a certain extent. The underlying mechanisms of how liraglutide improves IPG and beta cell function are obscured. Using a ZDF rat model in a pair-feeding experiment, Sturis *et al.* [32] found that 8 days treatment with liraglutide in rats with beta-cell deficiencies resulted in a significantly lower glucose excursion in response to oral glucose compared to vehicle treatment. Therefore, they believed that part of the antihyperglycemic effect of liraglutide was due to reduced food intake. However, this is in contrast to our findings, in which the FPG level was not related to bodyweight gain and food intake. Wang and Brubaker used the GLP-1 analogue Exendin-4 to treat 6-week old db/db mice and found that Exendin-4 treatment delayed the onset of diabetes through a mechanism involving Akt1 and expansion of the functional beta-cell mass [33]. In the present study, we found that a decreased antiapoptotic factor Bcl-2 expression and an increased proapoptotic factor Bax expression in pancreatic islets of vehicle-treated OLETF rats were reversed by12-week liraglutide treatment; meanwhile the abnormal islet structure was preserved by liraglutide treatment. We therefore speculate that liraglutide may protect islet formation and function through the regulation of apoptotic pathways, thereby reversing IFG and suppressing the diabetes onset.

Apart from protecting islet formation and function, liraglutide may also prevent the overt diabetes by inhibiting lipid metabolism and inflammatory reactions, thereby improving insulin resistance. Previous studies have shown that increased triglyceride levels, decreased high-density lipoprotein cholesterol (HDL-c) levels, and increased inflammatory markers can predict the progression to T2DM [34, 35]. Increased adipose tissue is a major factor to induce insulin resistance in obesity through the release of biochemical agents and inflammatory cytokines [36-38], and inflammatory cytokines may interfere with islet β-cell proliferation in a synergistic and glucose-independent manner [37]. Man *et al.* also reported that impaired beta-cell function and deposition of fat droplets in the pancreas could be a consequence of hypertriglyceridemia in OLETF rats [39]. Therefore, a treatment targeting high levels of lipid metabolism and inflammatory reactions in prediabetic subjects may dramatically preserve beta-cell function and prevent diabetes. In the present study, we found liraglutide treatment significantly reduced serum levels of TC and inflammatory markers such as Hs-CRP, IL-6, TNFα and PAI-1 in OLETF rats. TG level was also significantly lower in liraglutide-treated rats than vehicle-treated controls. However, LDL and HDL lev-

els as well as their ratio (data not shown) were not different between OLETF rats and normal controls before and after treatment. This may be related to their similar amount of food intake. Raun *et al.* also indicated that liraglutide improved insulin sensitivity and secretion, probably *via* reducing fat mass, thereby reducing circulating or islet triglycerides [40]. To our knowledge, this is the first report regarding the suppression of serum levels of inflammatory markers by liraglutide in prediabetic animals. Inflammation is closely related to cardiovascular complications in diabetes. The favorable effect of liraglutide on inflammatory status may help to prevent cardiovascular pathologies from the prediabetes stage. It is unknown how liraglutide inhibits inflammatory reaction. *In vitro* study has shown that liraglutide can dose-dependently inhibit NF-κB activation and TNFα-induced IκB degradation in human umbilical vein endothelial cells [41]. We suppose the suppression of inflammatory markers by liraglutide partly results from the reduction of body weight and lipid metabolism because a positive correlation was found between the bodyweight gain and serum level of Hs-CRP, and between the serum level of TG and PAI-1. Further studies are needed.

According to FDA's guidance [25], liraglutide 50μg/kg twice daily in rats can be converted to nearly 1 mg/day in humans (assuming human bodyweight as 60kg). Our results have shown that liraglutide can improve glycemic metabolism and insulin sensitivity as well as inflammatory status with a dose as low as 50μg/kg twice daily, which is similar to commonly used dosage of liraglutide clinically. Therefore, our results can be implicated as a proper reference for clinical practice.

Some limitations remain in the present study. A control group of LETO rats with liraglutide treatment has not been included in the study, which may cause some concerns about the effect of liraglutide on body weight and serum glucose level in LETO rats. There was not any report about the liraglutide treatment on LETO rats previously. A double-blind trial in 24 healthy Japanese men by Irie *et al.* [42] demonstrated that liraglutide could decrease mean and postprandial plasma glucose in dose dependent manner but all values remained within normal ranges. Although there was a tendency for weight to decrease with liraglutide in comparison to placebo, it is not significant. Clinical studies also show that liraglutide increases insulin production in a glucose-dependent manner [14, 43]. We can speculate that liraglutide treatment may lead to limited changes in body weight, and blood glucose and insulin levels in LETO rats after meal. But our purpose is to investigate the effect of liraglutide on prediabetes mainly by comparing its effect on OLETF rats before and after treatment. Therefore, omitting the LETO liraglutide group won't have a big impact on the conclusion of the present study. In addition, the semiquantative measurement of Bcl-2 and Bax by immunohistochemistry is not as accurate as that by Western blot. But it can still indicate the significant changes to a certain extent and also provide information about the expression localization in cells. Nevertheless, this is the first study to systemically investigate the effect of liraglutide in prediabetic OLETF rats, providing useful information for the application of liraglutide clinically.

In conclusion, chronic liraglutide administration, through its actions on glucose homeostasis by various mechanisms, resulted in highly efficacious diabetes prevention. Our findings suggest that a long-term treatment with liraglutide in prediabetic animals can not only prevent the onset of diabetes but also reverse hyperlipideamia and a high inflammatory status.

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Contribution Statement

We declare that all the listed authors have participated actively in the study and all meet the requirements of the authorship. Dehong Cai, Nanjing Guo and Jia Sun designed the study and wrote the protocol. Nanjing Guo and Zhen Zhang completed the study. Hong Chen and Jia Sun contributed to the literature search. Hua Zhang undertook the statistical analysis and Nanjing Guo wrote the draft of the manuscript.

Disclosure Statement

The authors have no conflicts of interest to declare.

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