



Characteristics of WBN/Kob diabetic fatty rats supplemented with a fructose-rich diet as a metabolic syndrome model: response to a GLP-1 receptor agonist

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ABSTRACT. The incidence of metabolic syndrome is rapidly increasing worldwide, and adequate animal models are crucial for studies on its pathogenesis and therapy. In the search of an adequate experimental model to simulate human metabolic syndrome, the present study was performed to examine the pharmacological response of WBN/Kob-*Lep^{fa}* (WBKDF) rats supplemented with a fructose-rich diet (FRD) to liraglutide, a GLP-1 receptor agonist. Male WBKDF rats fed FRD at 7 weeks of age were divided into 3 groups, and administered liraglutide (75, 300 µg/kg subcutaneously) or saline (control group), once daily for 4 weeks. All rats in the control group became overweight, and developed hyperglycemia, hypertension and dyslipidemia as they aged. The rats given liraglutide exhibited a dose-dependent reduction in body weight, visceral fat content and food intake compared with control rats. In addition, liraglutide suppressed the development of hyperglycemia, hypertension and dyslipidemia. An intravenous glucose tolerance test revealed that liraglutide improved glucose tolerance, insulin secretion and insulin resistance. On histological examination, decreased hepatic fatty degeneration was observed in the liraglutide groups. The present study demonstrated that liraglutide protected against obesity, hyperglycemia, hypertension, dyslipidemia, and hepatic steatosis in WBKDF rats fed FRD, suggesting that WBKDF rats fed FRD may be a useful model to investigate the etiology of human metabolic syndrome.

KEY WORDS: diet-induced obesity, liraglutide, metabolic syndrome, WBN/Kob-*Lep^{fa}* rat

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Metabolic syndrome (MS) is composed of several well-established risk factors such as obesity, hyperglycemia, insulin resistance, hypertension, dyslipidemia and/or non-alcoholic fatty liver disease [34]. These risk factors observed during MS are associated with the development of type 2 diabetes mellitus (T2DM) and cardiovascular disease [14, 23]. Therefore, these risk factors need to be aggressively treated in order to prevent overt type 2 diabetes and cardiovascular disease [34].

Liraglutide, a glucagon-like peptide-1 (GLP-1) receptor agonist, is an established anti-diabetic agent that is safe and effective for glycemic control [11, 22, 33]. In addition to its effective and safe glucose-lowering activity, it has been reported that GLP-1 analogs ameliorate glucose homeostasis by inhibiting endogenous glucagon production, suppress appetite and improve insulin resistance [12]. Furthermore, they also have favorable effects on several metabolic pathways, including body weight, blood pressure and lipid profiles, over and above their blood glucose lowering action [4, 5, 10, 25, 38, 40]. GLP-1 analogs are also expected to exert cardio-protective effects due to their effective glucose-lowering activity and favorable potency on multifactorial metabolic pathways [16].

An increase in MS patients worldwide has stimulated the development of experimental models. However, it is still challenging to find a dietetic model that closely approximates human MS. Based on the polygenic nature of human MS, studies examining monogenic or pharmacologically induced obesity models must be interpreted with care. The Wistar Bonn Koberi (WBN/Kob) diabetic fatty (WBKDF) rat is a new congenic strain with the leptin receptor fatty gene (*Lep^{fa}*), a recessive mutation that leads to leptin receptor deficiency and hyperphagia [7]. Previous reports found that WBKDF rats develop obesity and insulin resistance, both of which lead to T2DM [1, 15, 26, 30]. In our previous studies, after a 4-week feeding of a fructose-rich diet (FRD), WBKDF rats exhibited aggravated obesity and dyslipidemia compared with WBKDF rats fed a standard diet, whereas severe hyperglycemia

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naturally occurring in WBKDF rats fed a standard diet was moderately suppressed in WBKDF rats fed FRD [28]. These data suggest that WBKDF-FRD rats may be a useful model to simulate human MS.

Although there are numerous published studies using experimental models of MS, the model that most closely approximates human MS has yet to be found. In the search of an adequate experimental model to simulate human MS, the present study was performed to examine the pharmacological response of WBKDF-FRD rats to liraglutide, a clinically-used GLP-1 receptor agonist for MS patients.

MATERIALS AND METHODS

Animals

Male WBKDF rats at 5 weeks of age were obtained from Japan SLC, Inc. (Hamamatsu, Japan). All rats were housed in controlled temperature ($21 \pm 2^\circ\text{C}$), humidity ($55 \pm 5\%$) and lighting (08:00–20:00 hr) conditions throughout the experiment. They were allowed free access to food and fresh tap water from a plastic water bottle. All experimental protocols were approved by the Azabu University Animal Research Ethics Committee.

Study protocols

After a week of habituation, the standard rat chow (SRC, catalog number: CE-2, CLEA Japan, Inc., Tokyo, Japan) was changed to a fructose-rich diet (FRD, 60% fructose purified diet, catalog number: 5375, PMI Nutrition International, St. Louis, MO, U.S.A.) at 6 weeks of age. WBKDF rats ($n=24$) at 7 weeks of age were randomly divided into three groups of eight rats each: a control group, a low dose ($75 \mu\text{g}/\text{kg}$) of liraglutide group and a high dose ($300 \mu\text{g}/\text{kg}$) of liraglutide group. The doses of liraglutide were selected based on results from previous studies [26, 27]. Saline or liraglutide (Victoza[®], purchased from Novo Nordisk Pharma Ltd., Tokyo, Japan) was administered subcutaneously once daily for 4 weeks. The body weight of the rats was measured weekly between 10:00 and 14:00 hr. Non-fasting plasma glucose levels were measured weekly using blood samples collected from the tail vein. Age-matched WBKDF rats fed the SRC ($n=8$) were used as intact controls for measurement of blood pressure and histopathological examination of the liver.

Measurement of blood pressure

Systolic blood pressure (SBP) was monitored using a tail cuff blood pressure analyzer (BP98A-L, Softron, Tokyo, Japan) as described previously [36]. The rats were prewarmed for 15–20 min at 32°C to improve the detection of pulsation of the tail artery. SBP was calculated from the mean value after 3 successive measurements without signal disturbances.

Intravenous glucose tolerance test (IVGTT)

The intravenous glucose tolerance test (IVGTT) was performed for all rats at 11 weeks of age. Animals were fasted overnight before the test and anesthetized with pentobarbital sodium ($50 \text{ mg}/\text{kg}$, plus maintenance doses if necessary) through intraperitoneal injection. Glucose (20% w/v; Otsuka Pharmaceutical, Tokyo, Japan) was injected into the femoral vein at a dose of $0.5 \text{ g}/\text{kg}$ of body weight. Blood samples (0.2 ml) were then collected from the jugular vein at time intervals of 0, 2, 5, 10, and 20 min after injection. Heparinized plasma was separated by centrifugation ($2,000 \times g$ for 15 min) to measure plasma glucose and insulin levels.

Insulin resistance was assessed by the homeostasis model assessment of insulin resistance (HOMA-IR), which was calculated using the fasting plasma glucose and insulin levels [24]. The area under the curves (AUCs) of plasma glucose and insulin during IVGTT in each group was derived using the trapezoidal rule and the differences between groups were compared.

Fat content

After the IVGTT, a blood sample (3 ml) for chemical analysis was collected from the abdominal vena cava into a heparinized tube, centrifuged at $3,000 \times g$ for 15 min at 4°C , and the plasma was obtained and flash-frozen for blood biomarker analyses. At the end of the experiment, the rats were sacrificed using a lethal dose of pentobarbital. The epididymal fat and mesenteric fat were then collected and weighed.

Blood biomarkers

Plasma levels of total cholesterol (T-Cho), phospholipid (PL), triglycerides (TG) and glucose were measured using an automatic analyzer (JCA-BM 2250; JEOL Ltd., Tokyo, Japan). Plasma insulin levels were quantitated with the rat insulin ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan).

Histopathological examination

For histopathological analysis, fragments of the liver were fixed in 10% phosphate-buffered paraformaldehyde and embedded in paraffin. Paraffin blocks were cut into $4\text{-}\mu\text{m}$ thick sections and stained with hematoxylin and eosin (H&E).

Statistical analysis

Values are expressed as the mean \pm standard error (SE) unless otherwise stated. Statistical analysis was performed by one-way ANOVA followed by *post-hoc* Dunnett's test. Significance was set at *P*-values less than 0.05. All statistical analyses were performed using GraphPad Prism 5 statistical software (GraphPad Software Inc., La Jolla, CA, U.S.A.).

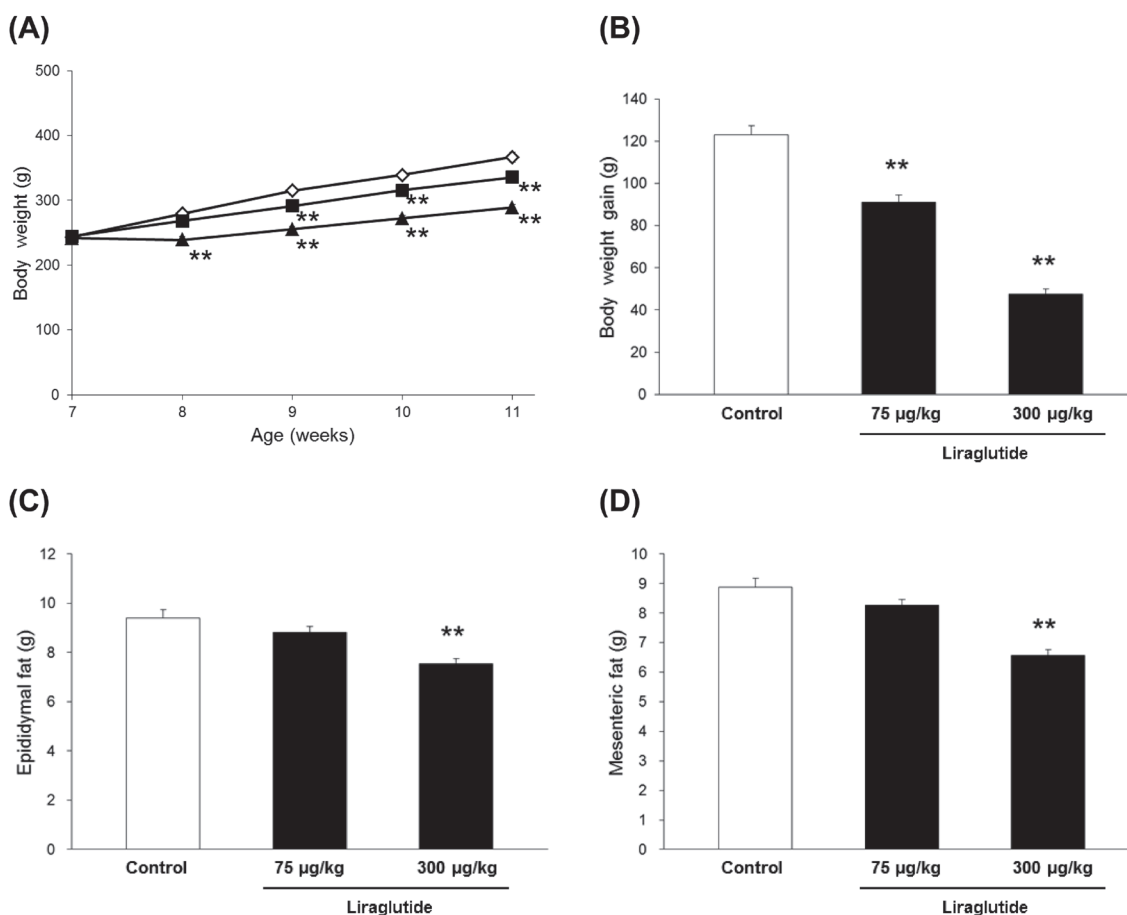


Fig. 1. Effects of low- and high-dose liraglutide on body weight (A) (open diamond, control; closed square, 75 µg/kg of liraglutide; closed triangle, 300 µg/kg of liraglutide), body weight gain (B), epididymal fat weight (C) and mesenteric fat weight (D) in WBKDF rats supplemented with a fructose-rich diet for 4 weeks. The fat weights were measured at 11 weeks of age. Data are expressed as the mean ± SE (n=8). Significant differences from the control group are indicated (**: $P < 0.01$, Dunnett's test).

RESULTS

Effects of liraglutide on body weight and fat weight

Prior to liraglutide intervention, there were no significant intergroup differences in body weight of WBKDF rats fed FRD (Fig. 1A). The body weight of the control group continued to increase during the experiment. The body weights in the liraglutide groups were reduced in a dose-dependent manner compared with those in the control group (Fig. 1A). The body weight gain during the treatment period was suppressed in liraglutide groups, and there was significant ($P < 0.01$) difference between the control and liraglutide groups (Fig. 1B). Furthermore, the epididymal and mesenteric fat weights in the high-dose liraglutide group were also significantly ($P < 0.01$) lower than those in the control group (Fig. 1C and 1D).

Effects of liraglutide on intake of food and water

No intergroup differences in the food and water intake of the 7-week-old rats were observed during the pre-administration period (Fig. 2A and 2B). Following drug administration, food and water intake in the liraglutide groups was suppressed in a dose-dependent manner, with significant differences observed after 8 weeks of age (Fig. 2A and 2B).

Effects of liraglutide on non-fasting blood glucose levels

No significant intergroup differences in non-fasting blood glucose levels were observed prior to the start of drug administration. The blood glucose levels in the control group (105 ± 4 mg/dl at 7 weeks of age) increased after 7 weeks of age, and at 11 weeks of age, a high blood glucose level of 333 ± 42 mg/dl was noted (Fig. 3). On the other hand, both liraglutide groups demonstrated complete suppression of hyperglycemia (Fig. 3).

Intravenous glucose tolerance test (IVGTT)

In the fasting state, plasma levels of glucose (75 µg/kg, $P < 0.05$; 300 µg/kg, $P < 0.01$) and insulin ($P < 0.05$) were significantly

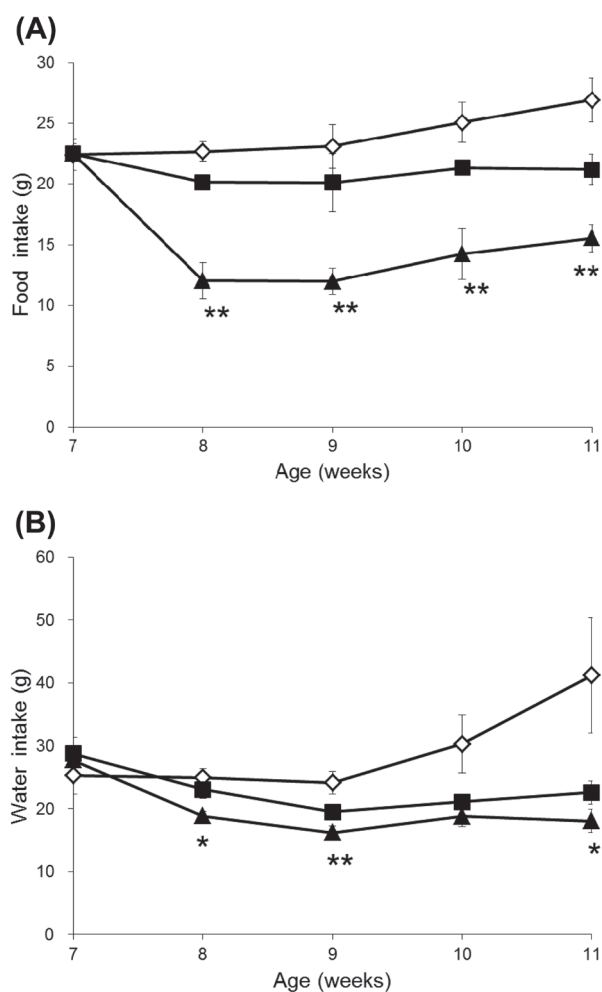


Fig. 2. Effects of low- and high-dose liraglutide on food intake (A) and water intake (B) in WBKDF rats supplemented with a fructose-rich diet for 4 weeks (open diamond, control; closed square, 75 µg/kg of liraglutide; closed triangle, 300 µg/kg of liraglutide). Data are expressed as the mean ± SE (n=8). Significant differences from the control group are indicated (*: $P<0.05$, **: $P<0.01$, Dunnett's test).

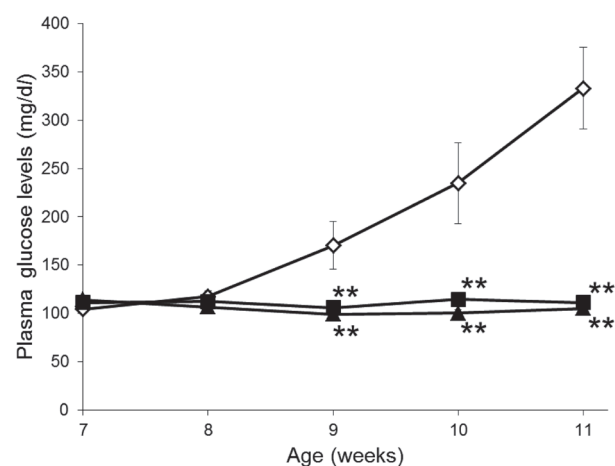


Fig. 3. Effects of low- and high-dose liraglutide on non-fasting plasma glucose concentrations in WBKDF rats supplemented with a fructose-rich diet for 4 weeks (open diamond, control; closed square, 75 µg/kg of liraglutide; closed triangle, 300 µg/kg of liraglutide). Data are expressed as the mean ± SE (n=8). Significant differences from the control group are indicated (**: $P<0.01$, Dunnett's test).

lower in the liraglutide groups than in the control group (Fig. 4A and 4B). As shown in Fig. 4A, the blood glucose levels at 20 min after the glucose load were significantly lower in the high-dose liraglutide group ($P<0.05$) than in the control group. As shown in Fig. 4B, plasma insulin levels increased in response to the glucose load in both the liraglutide and control groups. Compared with the control group, the liraglutide groups consistently had significantly higher blood plasma insulin concentrations after the glucose load. The plasma glucose and insulin levels measured periodically during IVGTT were used to calculate AUCs as indexes of glucose intolerance and insulin secretion, respectively. The glucose AUC was significantly reduced by 14% ($P<0.05$) in the high-dose liraglutide group compared with that in the control group (Fig. 4C). The insulin AUC was increased by 17% in the high-dose liraglutide group compared with that in the control group (Fig. 4D). HOMA-IR, which evaluates insulin sensitivity, was significantly lower ($P<0.05$) in the liraglutide groups than in the control group, suggesting improvement of insulin sensitivity in the liraglutide groups (Fig. 4E).

Effects of liraglutide on blood pressure

There was no significant difference in SBP among all three groups of WBKDF-FRD rats at 7 weeks of age (123 ± 5 , 125 ± 3 and 122 ± 4 mmHg in the control, low-dose and high-dose liraglutide groups, respectively). SBP in the control group at 11 weeks of age was higher than that at 7 weeks of age (123 ± 5 mmHg at 7 weeks of age and 139 ± 6 mmHg at 11 weeks of age). Liraglutide-treated rats at 11 weeks of age had lower values of SBP than those in the control group, and a significant difference ($P<0.01$) was observed between the high-dose liraglutide group and control group (Table 1). The blood pressure of WBKDF rats fed SRC was 121 ± 4 and 126 ± 3 mmHg at 7 weeks and 11 weeks of age, respectively.

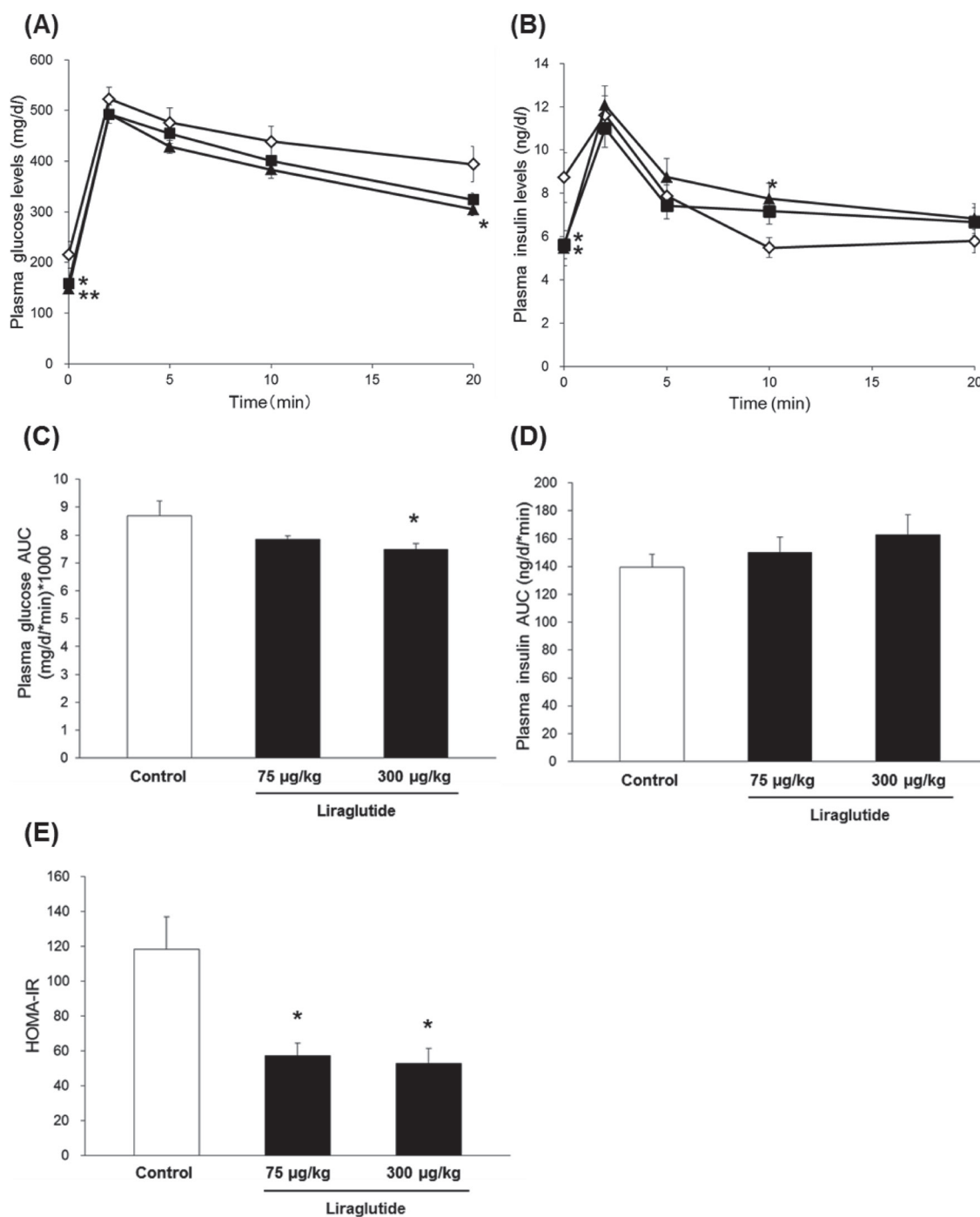


Fig. 4. Effects of low- and high-dose liraglutide on glucose tolerance in WBKDF rats supplemented with a fructose-rich diet for 4 weeks. The IVGTT was performed as described in the Materials and Methods section. The plasma levels of glucose (A) and insulin (B) during IVGTT were measured at 11 weeks of age (open diamond, control; closed square, 75 µg/kg of liraglutide; closed triangle, 300 µg/kg of liraglutide). The AUCs of plasma glucose (C) and plasma insulin (D) were calculated. The HOMA-IR (E) was calculated as described in the Materials and Methods section. Data are expressed as the mean ± SE (n=8). Significant differences from the control group are indicated (*: $P < 0.05$, **: $P < 0.01$, Dunnett's test).

Effects of liraglutide on plasma lipids

Based on the biochemical analyses, high-dose liraglutide-treated rats had significantly ($P < 0.01$) lower levels of T-Chol and PL than control rats (Table 1). However, there were no significant changes in TG (Table 1).

Effects of liraglutide on the liver

The liver weight in the liraglutide groups was significantly ($P < 0.01$) reduced in a dose-dependent manner compared with that in

Table 1. Effects of low- and high-dose liraglutide on blood pressure, plasma lipids and liver weight in WBKDF rats supplemented with a fructose-rich diet for 4 weeks

| Variables | Control | Liraglutide | |
|--|----------------|------------------------------|-----------------------------|
| | | 75 $\mu\text{g}/\text{kg}$ | 300 $\mu\text{g}/\text{kg}$ |
| Systolic blood pressure (mmHg) | 139 \pm 6 | 132 \pm 6 | 116 \pm 2 ^{a)} |
| Plasma T-Cho (mg/dl) | 184 \pm 3 | 179 \pm 4 | 144 \pm 7 ^{a)} |
| Plasma PL (mg/dl) | 344 \pm 6 | 341 \pm 12 | 295 \pm 14 ^{a)} |
| Plasma TG (mg/dl) | 517 \pm 73 | 484 \pm 52 | 493 \pm 56 |
| Absolute liver weight (g) | 24.8 \pm 2.2 | 12.6 \pm 0.7 ^{a)} | 9.5 \pm 0.3 ^{a)} |
| Ratio of liver weight to body weight (%) | 7.2 \pm 0.8 | 3.9 \pm 0.1 ^{a)} | 3.4 \pm 0.1 ^{a)} |

Data are expressed as the mean \pm SE (n=8). T-Cho: total cholesterol, PL: phospholipid, TG: triglycerides. Significant differences from the control group are indicated (a: $P < 0.01$, Dunnett's test).

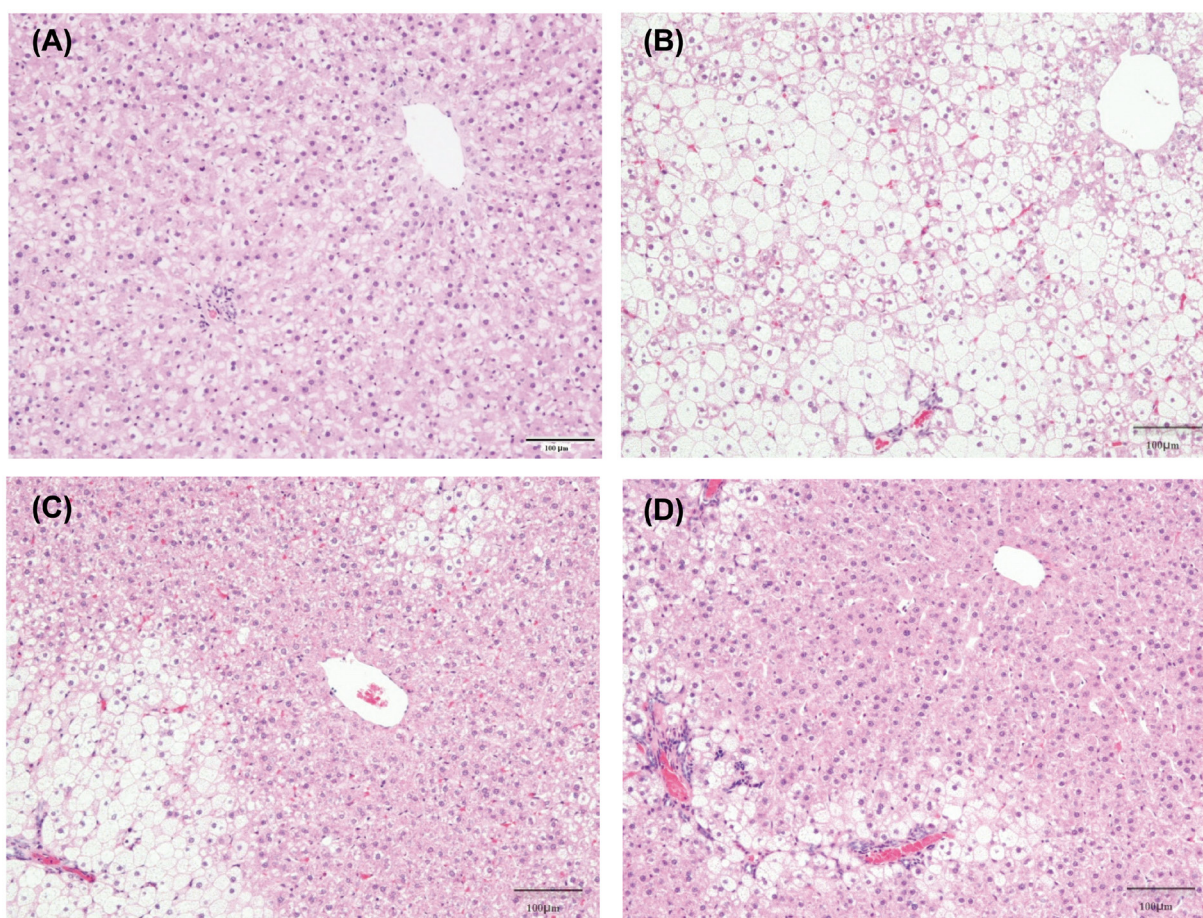


Fig. 5. Effects of low- and high-dose liraglutide on livers of WBKDF rats supplemented with a fructose-rich diet for 4 weeks. Histopathological examination [WBKDF rats fed standard rat chow (A), control (B), 75 $\mu\text{g}/\text{kg}$ of liraglutide (C) and 300 $\mu\text{g}/\text{kg}$ of liraglutide (D)] was performed as described in the Materials and Methods section.

the control group (Table 1). Histopathological evaluation of H&E stained liver sections revealed marked diffuse fatty degeneration in the control group compared with that in WBKDF rats fed SRC (Fig. 5A and 5B). The fatty degeneration in the liraglutide groups was reduced in a dose-dependent manner compared with that in the control group (Fig. 5C and 5D).

DISCUSSION

The incidence of MS is increasing rapidly worldwide, and adequate animal models are crucial for studies on its pathogenesis and therapy. In the search for an adequate experimental model that simulates human MS, the present study was performed to examine the pharmacological response of WBKDF-FRD rats to liraglutide, a clinically-used GLP-1 receptor agonist for MS patients.

As in our previous study, the feeding of FRD resulted in several features of MS, including glucose tolerance, obesity, and dyslipidemia, in WBKDF rats. Furthermore, hypertension and hepatic steatosis were also noted in WBKDF-FRD rats. Liraglutide given daily for 4 weeks significantly improved glucose tolerance, obesity, hypertension, dyslipidemia, and hepatic steatosis associated with MS in WBKDF-FRD rats.

To develop an animal model mimicking the structural and functional features of MS in patients with T2DM, we fed WBKDF rats with FRD [28]. As FRD-fed rats have been used as an experimental model of human conditions [29], data on the pathological effects of a high fructose intake in experimental models is abundant. For example, fructose produces high plasma levels of insulin, glucose, cholesterol and triglycerides in rats [8, 13, 17, 19]. However, the metabolic alterations observed in fructose-fed rats vary among studies, likely due to the experimental design [6, 35]. Differences among studies include the strain and the age of rats, and the amount, period and route of fructose administration. Consistent with our previous study [28], WBKDF-FRD rats exhibited signs of human MS, including glucose tolerance, obesity, hypertension, dyslipidemia and hepatic steatosis. Taken together, these alterations confirmed the proper induction of MS in our study.

Our previous study [28] demonstrated that FRD feeding moderately attenuated the severe hyperglycemia in contrast to high-fat diet, which aggravated hyperglycemia in WBKDF rats. In the present study, liraglutide completely suppressed hyperglycemia in the WBKDF-FRD rats. In addition, HOMA-IR was significantly lower in the liraglutide groups than in the control group, suggesting that liraglutide improved insulin sensitivity. These results are consistent with previous clinical studies reporting potent anti-diabetic effects of liraglutide [11, 12, 22, 33], which are mainly due to its insulinotropic and anti-hyperphagia activity.

WBKDF rats carry the fatty mutation (*fa*) in the leptin receptor gene and spontaneously develop obesity. The present study demonstrated that FRD loading aggravated obesity that developed spontaneously in WBKDF rats, which is consistent with the results of our previous study [28]. Furthermore, liraglutide decreased the body weights and visceral fat amounts in the WBKDF-FRD rats. Similarly, clinical trials revealed that liraglutide decreased the body weights in overweight/obese patients with comorbid T2DM [32, 39]. The mechanism by which GLP-1 receptor agonists induce weight loss is believed to be related to multiple actions involving the brain and gastrointestinal tract, with the primary action related to an increase in satiety [31], which is consistent with the reduced food intake observed in the liraglutide groups in the present study. The water intake was also reduced by liraglutide in a dose-dependent manner. The reduced water intake by liraglutide was likely due to a secondary phenomenon of T2DM prevention.

FRD was reported to induce an increase in SBP [6] and mean arterial pressure in rats [18]. Similarly, a mild but significant elevation of SBP was observed in WBKDF-FRD rats, and this elevation of SBP was inhibited by liraglutide. The findings from this study are consistent with clinical trials in which liraglutide reduced SBP in patients with T2DM [9]. Kim *et al.* [21] demonstrated that cardiac GLP-1 receptor expression is localized to cardiac atria and that GLP-1 receptor agonists promote the release of atrial natriuretic peptide (ANP) and reduce blood pressure.

It was previously reported that rats fed FRD have characteristics of nonalcoholic hepatic steatosis [20]. Diffuse fatty degeneration along with high plasma levels of T-Chol, PL and TG were observed in control WBKDF-FRD rats in the present study. Thus, WBKDF-FRD rats exhibit fat deposition in the liver in addition to important changes characteristic of MS such as obesity, hyperglycemia and hyperlipidemia. Liraglutide was found to decrease fatty degeneration in the liver and plasma lipids, such as T-Chol and PL, in the WBKDF-FRD rats. Similarly, liraglutide reduced hepatic steatosis in patients with non-alcoholic steatohepatitis [3]. In addition, Armstrong *et al.* [2] demonstrated that liraglutide improved both hepatic and global/localized adipose insulin sensitivity, thereby reducing the amount of lipotoxic metabolites and pro-inflammatory mediators in circulation, and reduced hepatic *de novo* lipogenesis *in vivo*, a key component of hepatic lipid accumulation in nonalcoholic hepatic steatosis. The mechanisms underlying the effects of GLP-1 receptor agonists on lipid metabolism involve the direct effects of GLP-1 on the hepatic and intestinal production of triglyceride-rich lipoproteins, the GLP-1-induced increase in the production and function of insulin, the activation of specific areas of the central nervous system, as well as increased peripheral utilization of triglycerides for energy production [37].

In the present study, liraglutide significantly improved all major metabolic parameters associated with MS, such as glucose tolerance, obesity, hypertension, dyslipidemia and hepatic steatosis, in WBKDF-FRD rats. Clinical trials revealed that liraglutide decreased the body weights, SBP and hepatic steatosis in patients with features of MS, suggesting that the pharmacological response of WBKDF-FRD rats to liraglutide is similar with that of human MS patients.

Concerning the limitations of the study, the lack of baseline values for the biochemical variables means that existing differences may have been missed. However, the randomization process to compose the groups diminished this possibility. The absence of physical activity measurements does not exclude the possibility that the anti-metabolic effects of liraglutide are related to lower physical or metabolic activity. Based on the randomization assumption, liraglutide may play a role in spontaneous physical activity. Further studies are required to confirm this possibility.

In summary, we examined the pharmacological response of WBKDF rats supplemented with FRD to liraglutide, a GLP-1 receptor agonist. Liraglutide significantly improved all major metabolic parameters associated with MS in WBKDF-FRD rats, suggesting that the pharmacological responses of WBKDF-FRD rats to liraglutide are similar with those of human MS patients. WBKDF-FRD rats may be a useful model to investigate the etiology of human MS.

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