Additive protection by LDR and FGF21 treatment against diabetic nephropathy in type 2 diabetes model

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Running Head: LDR plus FGF21 prevent diabetic nephropathy

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Abstract

The onset of diabetic nephropathy (DN) is associated with both systemic and renal changes. Fibroblast growth factor (FGF)-21 prevents diabetic complications mainly by improving systemic metabolism. In addition, low-dose radiation (LDR) protects mice from DN directly by preventing renal oxidative stress and inflammation. In the current study, we tried to define whether the combination of FGF21 and LDR could further prevent DN by blocking its systemic and renal pathogeneses. To this end, type 2 diabetes was induced by feeding a high-fat diet for 12 weeks followed by a single dose injection of streptozotocin. Diabetic mice were exposed to 50 mGy LDR every other day for 4 weeks with and without 1.5 mg/kg FGF21 daily for 8 weeks. The changes in systemic parameters, including blood glucose levels, lipid profiles, and insulin resistance, as well as renal pathology were examined. Diabetic mice exhibited renal dysfunction and pathological abnormalities, all of which were prevented significantly by LDR and/or FGF21; the best effects were observed in the group that received the combination treatment. Our studies revealed that the additive renal protection conferred by the combined treatment against diabetes-induced renal fibrosis, inflammation, and oxidative damage was associated with the systemic improvement of hyperglycemia, hyperlipidemia, and insulin resistance. These results suggest that the combination treatment with LDR and FGF21 prevented DN more efficiently than did either treatment alone. The mechanism behind these protective effects could be attributed to the suppression of both systemic and renal pathways.

Key words: low dose radiation, fibroblast growth factor-21, insulin resistance, inflammation, oxidative stress
Introduction

Diabetic nephropathy (DN) is a severe complication of diabetes and the leading cause of end-stage renal disease (38). DN initiates with the thickening of the glomerular basement membrane, which is followed by mild and moderate mesangial expansion, capillary collapse in the renal tubule, epithelial cell degeneration, and a gradual increase in proteinuria, and finally leads to renal fibrosis and kidney failure (43, 56). Hyperglycemia, hyperlipidemia, and subsequent insulin resistance are the initial and systemic pathogeneses of DN, which is also associated with renal pathogeneses, such as oxidative stress, inflammation, and fibrosis.

There are currently multiple therapies to treat patients with DN, including reducing blood sugar levels, lowering lipid levels, enhancing insulin sensitivity, and reducing oxidative stress, inflammation, or fibrosis (1, 19, 50). However, most of these treatments just slow rather than arrest the progression toward DN since blocking any single pathway is not sufficient to prevent the development of DN (8, 19). Therefore, identifying a treatment that inhibits both the systemic and renal pathogeneses will prevent the development of DN efficiently.

Fibroblast growth factor (FGF)-21 is a novel member of FGF family that functions as an endocrine hormone rather than to regulate cellular proliferation and differentiation (3, 29). FGF21 acts on multiple tissues to regulate carbohydrate and lipid metabolism. Large amounts of evidence has demonstrated that FGF21 also induces beneficial effects in diabetes and its related complications by lowering blood glucose levels, stimulating lipid β-oxidation, and enhancing insulin sensitivity (25, 26). In addition, FGF21 protects the heart against oxidative stress-induced damage in two different mice models (isoproterenol-induced cardiac hypertrophy (40) and the LPS-induced pro-inflammatory pathway (7) by inducing the
expression of antioxidant proteins, including uncoupling proteins and SOD-2 (41). FGF21 also protects the liver from d-gal-induced oxidative stress by activating Nrf2 and subsequent antioxidant genes (54). Moreover, Kim et al. (27) demonstrated that FGF21 protects the kidneys in a mouse model of type 2 diabetes by improving insulin sensitivity. Our previous study also demonstrated the beneficial effect of FGF21 on type 1 diabetes-induced renal damage (56).

Low-dose radiation (LDR) generally refers to radiation at a dose less than 100 mGy for low linear energy transfer (LET) (32), which induces beneficial effects, including stimulating DNA/RNA repair, increasing the cellular antioxidant capacity, prolonging life span, and activating immune functions (13, 14). These cellular hormetic effects of LDR protect cells in vitro or tissues in vivo against gene mutations, DNA damage, and the chromosomal aberrations caused by subsequent large doses of radiation or toxic chemicals via an adaptive response (9, 10, 12). Increasing evidence has suggested that LDR reduces the incidence of diabetes and also exerts beneficial effects on diabetic complications such as DN (35, 45). In addition, our previous studies showed that exposure to LDR greatly prevented type 2 diabetes-induced renal injury, and also inhibited renal oxidative stress, inflammation, and fibrosis without altering blood glucose levels (43). We further demonstrated that LDR could increase Akt phosphorylation and the expression of Nrf2 and its downstream antioxidants significantly (51, 57).

Taken together, the above evidence suggests that both FGF21 and LDR induce beneficial effects on DN. FGF21 might mainly suppress the systemic pathogenesis, whereas LDR might mainly protect the kidneys from diabetes via anti-inflammatory, anti-oxidative, and
anti-fibrotic mechanisms (51, 56, 57). Therefore, the aim of the present study was to investigate whether the combination treatment with LDR and FGF21 could exert additive effects on the prevention of DN via both systemic and renal mechanisms.

Materials and Methods

Ethics statement

Eight-week-old male C57BL/6J mice (weighting 18–22 g) were purchased from the Experimental Animal Center of Beijing University of Medical Science (Beijing, China) and allowed to acclimate for 2 weeks. All mice were housed in the Experimental Animal Center of Jilin University at 22°C with 12:12-h light-dark cycles and free access to rodent chow and tap water. The animal production license is No. SCXK 2007-0003. All animal procedures were approved by the University Animal Care and Use Committee (permit number 2007-0011), which is certified by the Chinese Association of Accreditation of Laboratory Animal Care.

Animals and type 2 diabetes model

The high-fat diet (HFD)/streptozotocin (STZ) protocol was applied to establish the model of type 2 diabetes (34, 43, 49). Mice were fed a HFD (40% of calories from fat; Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) for 12 weeks to induce metabolic syndrome, as characterized by obesity, abnormal glucose tolerance, and insulin resistance. Age-matched non-diabetic mice were fed a standard diet (SD, 10% of calories from fat; Shanghai SLAC Laboratory Animal Co.). The obese mice were divided randomly into two groups: one group was given a single i.p. injection of 50 mg/kg body-weight STZ to
induce hyperglycemia and the onset of type 2 diabetes, whereas the other group was given an i.p. injection of an equivalent volume of citrate buffer (Con). Mice were considered diabetic when their blood glucose exceeded 12 mmol/L. Subsequently, the diabetic mice were divided into 3 sub-groups: diabetes mellitus (DM) plus LDR treatment (DM/LDR), DM plus FGF21 treatment (DM/FGF21), and DM plus the combination of LDR and FGF21 treatment (DM/Com).

**Whole-body LDR and FGF21 treatment**

A 180-kVp X-ray generator (Model XSZ-Z20/20, China) was used to deliver radiation at a dose rate of 12.5 mGy/min (120 kv, 13 mA). Diabetic mice in the DM/LDR and DM/Com groups received whole-body irradiation every 2 days at a dose of 50 mGy/day for 4 weeks, based on our previous observations (56). Mice in the DM/FGF21 or DM/Com groups were given i.p. injections of 1.5 mg/kg FGF21 daily for 8 weeks. Therefore, the DM/Com group received both LDR and FGF21 treatment for 4 weeks followed by an additional 4 weeks of FGF21 treatment alone.

*Glucose tolerance test (GTT) and insulin tolerance test (ITT)*

To evaluate glucose tolerance, mice were given an i.p. injection of D-glucose (1.5 g/kg) after an overnight fast (12 h) with free access to water. Venous blood was collected 30 min before the injection of glucose (time 0), as well as 15, 30, 60, and 120 min after injection from the tail of each mouse, and glucose levels were measured using a OneTouch SureStep complete blood glucose monitor (LifeScan, Milpitas, CA, USA). To evaluate insulin tolerance, a single dose of 0.5 U/kg Novolin R regular insulin (Novo Nordisk A/S, Bagsvaerd, Denmark) was administered intraperitoneally after a 4-h fast with free access to water, and
the blood glucose levels were measured as described above.

**Measuring renal function**

Mice were placed in metabolic cages individually with free access to tap water on the day before sacrifice to collect 24-h urine samples. Total urinary protein (UP), urinary microalbumin (mAlb), and urinary creatinine (U-Cre) contents were measured as parameters of renal function using enzyme-linked immunosorbent assay (ELISA) kits purchased from R&D Systems (Itasea, MN, USA) according to the manufacturer’s instructions.

**Measuring serum and renal lipid profiles**

Mice were sacrificed after anesthetized, and blood was collected by cardiac puncture and centrifuged at $2,000 \times g$ for 20 min at $4^\circ C$ to prepare plasma. Plasma triglycerides (TG), total cholesterol (CHO), high-density lipoprotein (HDL)-cholesterol, and low-density lipoprotein (LDL)-cholesterol levels were determined using an Olympus Au800 automatic biochemical analyzer (Olympus, Japan).

To measure TG and free fatty acid (FFA) contents in the kidney, ~80 mg of kidney tissue from each mouse was homogenized in 250 μL of buffer containing 150 mM NaCl and 10 mM Tris (pH 7.5), and then extracted using 200 μL of methanol and 400 μL of chloroform, as described by Bligh and Dyer (6). The rest of the procedure was same as that described above using an Olympus Au800 automatic biochemical analyzer (Olympus).

**Total protein extraction and western blotting**

Renal tissues were homogenized in lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the supernatants were collected by centrifugation at $12,000 \times g$ and $4^\circ C$. After determining the total protein concentration, equal amounts of each sample were run on 10%
SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After blocking with non-fat milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with the following primary antibodies: nuclear factor E2-related factor-2 (Nrf-2, 1:1000), superoxide dismutase-1 (SOD-1, 1:2000), NAD(P)H:quinone oxidoreductase-1 (NQO-1, 1:1000), heme oxygenase-1 (HO-1, 1:2000), 3-nitrotyrosine (3-NT, 1:1000), intercellular adhesion molecule-1 (ICAM-1, 1:2000), plasminogen activator inhibitor-1 (PAI-1, 1:2000), connective tissue growth factor (CTGF, 1:2000), tumor necrosis factor-α (TNF-α, 1:1000), and β-actin (1:2000). All antibodies were purchased from Abcam (Cambridge, MA, USA), except for β-actin (Cell Signaling Technology, Danvers, MA, USA). After three washes in Tris-buffered saline containing 0.05% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Antigen-antibody complexes were then visualized using an enhanced chemiluminescence kit (Amersham, Piscataway, NJ, USA), and the intensity of the protein bands was quantified using Quantity one software (Version 4.6.2, Bio-Rad, USA).

**Histological examination**

Kidney tissues were fixed in 10% formalin at room temperature for 48 h. After dehydration, the tissue blocks were embedded in paraffin and cut into 4-mm-thick sections. They were then stained with hematoxylin and eosin (H&E) for general morphological examination, periodic acid-Schiff (PAS) for glomerulosclerosis evaluation, and Masson’s Trichrome staining for interstitial expansion assessment, as described previously (24, 44, 56). Five different fields were selected randomly from each slice, each containing at least 50 glomeruli. The degree of glomerulosclerosis was divided into five grades from 0 to 4.
according to the number of sclerotic lesions in each glomerulus (grade 0, normal glomerulus; grade 1, sclerotic area of 1–25%; grade 2, sclerotic area of 26–50%; grade 3, sclerotic area of 51–75%; grade 4, sclerotic area of 76–100%). The glomerulosclerosis index (GSI) was calculated using the following formula:

$$\text{GSI} = \frac{(N_1 \times 1) + (N_2 \times 2) + (N_3 \times 3) + (N_4 \times 4)}{N_{\text{total}}},$$

where $N$ represents the number of the corresponding degree of glomerulosclerosis and $N_{\text{total}}$ is the total number of glomeruli. Similarly, the extent of interstitial expansion was quantified by calculating the tubular interstitial collagen-positive area (blue) of Masson’s Trichrome staining using Image-Pro plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Twenty consecutive glomeruli were examined for each section, and the mean percentage of collagen-positive lesions was calculated for each mouse.

**Assaying lipid oxidation**

A thiobarbituric acid (TBA) assay was used to measure relative malondialdehyde (MDA) production as an index of lipid peroxidation, as described previously (11). Briefly, tissue proteins were collected by centrifuging at $12,000 \times g$ at 4°C for 15 min, and the protein concentrations were measured using Bradford assays. Then, 50 μl of sample was mixed with 20 μl of 8.1% sodium dodecyl sulfate (SDS), 150 μl of 20% acetic acid, and 210 μl of 0.0571% TBA, and was incubated at 90°C for 70 min. Samples were centrifuged at 4000 rpm for 15 min at 4°C, harvested, transferred to 96-well plates, the optical density was read at 540 nm.

**Statistical analysis**

Data were collected from nine mice in each group, and the results are presented as
means ± standard error of the mean (SEM). Statistical analyses were performed using one-way or two-way analysis of variance (ANOVA), followed by post-hoc multiple comparisons using Bonferroni’s tests. All analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as $P < 0.05$.

**Results**

**Type 2 diabetic mouse model**

Clinically, the major features of type 2 diabetes are insulin resistance together with obesity, hyperlipidemia, and hyperglycemia. Therefore, in the current study mice were fed a HFD to induce obesity, hyperlipidemia, and insulin resistance, and were then treated using a single injection of STZ to induce hyperglycemia and establish a model of type 2 diabetes. As shown in Fig. 1, there was a significant increase in their body-weight starting 4 weeks after HFD feeding compared with SD feeding (Con), and the difference between two groups increased gradually in a time-dependent manner (Fig. 1A). In the GTT, the blood glucose level in the HFD-fed mice after glucose infusion was greater than that in the SD-fed mice at 30 min, suggesting that the HFD-fed mice were glucose intolerant (Fig. 1B). In addition, the blood glucose level in the HFD-fed mice remained higher than did that in the SD-fed mice until the final measurement, although there were no statistically significant differences between the two groups (Fig. 1B). Furthermore, blood glucose levels during the ITT were significant higher in HFD-fed mice than in SD-fed mice at all time points (Fig. 1C). The above results suggest that the mice fed with a HFD for 12 weeks had already developed glucose intolerance and insulin resistance (Fig. 1B, C). A single injection of STZ induced a
significant increase in blood glucose compared with the HFD-fed alone group, but did not
induce hyperglycemia compared with the SD-fed (Con) group (Fig. 1D).

*The combination of LDR and FGF21 improved insulin sensitivity in type 2 diabetic mice significantly compared with treatment alone*

Diabetic mice were fed a HFD in the presence of FGF21 alone, LDR alone, or the combination of both treatments. At the end of experiment, FGF21 and the combination treatment, not LDR, decreased blood glucose levels significantly compared with the DM group (Fig. 2A). Although the blood glucose levels in the combination group were decreased slightly compared with FGF21 treatment alone, the difference was not statistically significant (Fig. 2A). In addition, both LDR and FGF21 significantly ameliorated diabetes-induced insulin resistance (Fig. 2B); however, the combination treatment improved insulin sensitivity additively compared with either single treatment alone (Fig. 2B).

*Effects of the different treatments on renal dysfunction in diabetic mice*

Diabetes increased the levels of UP and mAlb significantly, indicating the induction of renal dysfunction (Table 1). However, FGF21 treatment prevented diabetes-induced renal dysfunction significantly, as shown by reduced UP and mAlb and increased U-Cre levels. Exposure to LDR also reduced UP and mAlb significantly, but did not affect U-Cre levels. Renal dysfunction which reflected by increased UP and mAlb levels, was diminished further in mice that received the combination treatment compared with either treatment alone. Both FGF21 alone and the combination treatment, but not LDR, increased U-Cre excretion significantly compared with the DM group (Table 1).

*Effects of the different treatments on diabetes-induced renal hypertrophy and pathological*
Generally, renal dysfunction reflects pathological changes in the kidney. Consistent with the functional findings (Table 1), mesangial cell proliferation, capillary wall thickness, capillary collapse, tubular dilation, and epithelial cell degeneration were all obvious in diabetic kidneys after H&E staining (Fig. 3A). These pathological changes were suppressed significantly by LDR or FGF21 treatment alone (Fig. 3A), and particularly by the combination treatment.

Next, the kidney tissues were stained with PAS to detect glomerulosclerosis. As shown in Figs. 3B and D, the glomerulosclerosis index was increased significantly in diabetic mice, which was prevented significantly by treatment with either LDR or FGF21 alone and to a greater extent by the combination treatment.

Masson’s trichrome staining revealed significant collagen accumulation in the kidneys of diabetic mice, indicating that interstitial fibrosis had developed (Figs. 3C, E). Treatment with either LDR or FGF21 alone prevented these pathological changes significantly, and the preventive effects were additive in the combination treatment group. To confirm these observations, we assessed CTGF expression in the kidneys as a classical molecular marker of fibrosis using western blotting. Consistent with the results of Masson’s staining, renal CTGF expression was increased significantly in the DM group, but not in the LDR-, FGF21-, or combination treatment DM groups (Fig. 3F).

Diabetes-induced kidney injury is always associated with renal hypertrophy, which is characterized by an increased kidney weight to tibia length ratio. In the current study, this ratio was increased in the DM group compared with HFD-fed mice group (Fig. 3G), but
decreased slightly after exposure to LDR. In contrast, treatment with FGF21 alone or in combination with LDR decreased the ratio significantly compared with the DM group (Fig. 3G).

**Effects of the different treatments on systemic and renal dyslipidemia in type 2 diabetic mice**

In addition to hyperglycemia, dyslipidemia is a crucial systemic pathogenesis of DN. As shown in Table 2, hyperlipidemia was evident in the DM groups, as shown by increased plasma TG levels. Consistent with the plasma profiles, kidney tissue H&E staining revealed an increased number of fat vacuoles in the kidney (Fig. 3A); the renal TG and FFA levels were also increased significantly in the diabetic mice (Table 2). These results suggest that renal lipid accumulation was increased in the DM group. However, treatment with FGF21 normalized all of the above abnormal profiles remarkably. Similar effects were observed in the DM/LDR mice, except that there was no change in CHO levels compared with the DM group. Importantly, the combination treatment normalized plasma and renal TG, plasma LDL, and renal FFA levels (Table 2), and also significantly reduced the number of renal fat vacuoles (Fig. 3A).

**Effects of the different treatments on diabetes-induced renal oxidative stress and inflammation**

As markers of nitrosative or oxidative damage, respectively, renal 3-NT (Fig. 4A) and MDA (Fig. 4B) levels were increased significantly in the DM group, but not in the DM/LDR, DM/FGF21, and DM/Com groups (Fig. 4A, B). To confirm these observations, western blotting was used to examine the levels of classic inflammatory factors in the kidney,
including ICAM-1 (Fig. 5A), TNF-α (Fig. 5B), and PAI-1 (Fig. 5C). Consistent with the above results, diabetes increased the expression of these inflammatory factors in the kidney significantly. However, the increase was prevented by treatment with LDR or FGF21 alone, and to an even greater extent by the combination treatment.

Effects of the different treatments on the renal expression of antioxidants in diabetic mice

Next, we measured the renal expression of multiple antioxidants in treated and untreated diabetic mice. Western blotting revealed that the expression of SOD-1 (Fig. 6A), NQO-1 (Fig. 6B), and HO-1 (Fig. 6C) was decreased significantly in the kidneys of DM mice and DM/FGF21 mice (Fig. 6A-C). Interestingly, exposure to LDR reversed the diabetes-induced suppression of these antioxidants significantly, and the expression of these antioxidants was much higher in the DM/LDR group than in the DM group (Fig. 6A-C). The expression of the antioxidants was similar in DM/Com and control mice. These results suggest that LDR, but not FGF-21, increased renal antioxidant levels significantly under diabetic conditions.

The expression of the abovementioned antioxidants is regulated positively by the transcription factor Nrf2. We demonstrated previously that Nrf2 is upregulated in diabetic mice exposed to LDR (51, 57). Therefore, we next determined whether the altered antioxidant expression in the DM/LDR and DM/Com groups was due to altered renal Nrf2 levels (Fig. 6D). As expected, the pattern of Nrf2 expression was similar to the antioxidant expression in the different groups, suggesting that diabetes downregulated and LDR upregulated renal antioxidant levels mainly by altering Nrf2 expression.
**Discussion**

The kidney is the main target organ of diabetes, which leads to the development of DN. DN is characterized by renal dysfunction and pathological changes such as renal hypertrophy, fibrosis, and glomerulosclerosis (18, 21, 33). The pathogenesis of DN is highly complex, and is the consequence of both systemic and renal pathogeneses. Therefore, we hypothesized that improving the systemic pathogenesis of diabetes while protecting the kidney from diabetes-induced damage might additively prevent the development of DN.

FGF21 is a member of the FGF family that exerts systemic therapeutic effects to improve hyperglycemia, dyslipidemia, and insulin resistance (2, 16). Serum FGF21 levels were increased significantly in humans with both acute and chronic renal dysfunction during early- to end-stage kidney disease, which might be an adaptive response to diabetes to confer protective effects (30). FGF21 suppresses diabetes-induced inflammation, oxidative stress, and fibrosis in multiple organs, which could be attributed to its effects on maintaining glucose and lipid homeostasis and improving insulin resistance (22, 27, 56). Similarly, renal protective effects of FGF21 were also observed in our recent study performed in an acute lipotoxic model of type 1 diabetes (56).

High doses of ionizing radiation (HDR) induce a variety of harmful effects, including acute death and late carcinogenesis. Although LDR was also considered dangerous according to the linear no-threshold (LNT) hypothesis (32), increasing evidence has suggested that LET radiation at dose levels lower than 100 mGy could induce beneficial effects, including extending life span, enhancing immunity, and improving DNA repair (17, 23, 36). Epidemiological surveys have analyzed individuals exposed to <100 mGy radiation.
Generally, there was (1) no increase or even a reduced risk of the incidence of solid cancer; (2) no increase in the incidence of leukemia; and (3) no increase in cardiovascular diseases (5, 15, 28, 31, 37). A study supported by the Radiation Effects Research foundation showed that the dose-response relationship supported the LNT model in principle in the dose range 0–150 mGy; however, the dose-response relationship below 100 mGy tends to fluctuate, which limits statistical significance in the increase in the incidence of cancer at lower doses (42). Our recent studies demonstrated that LDR also induces beneficial effects on diabetes and its associated complications (43, 56, 57). The available data suggest that pre-exposure to LDR reduces the incidence of alloxan-induced diabetes, and also delays the onset of hyperglycemia in diabetes-prone non-obese diabetic mice (46, 48). This protection is associated with the prevention of oxidative damage (51-53, 57). Our previous study revealed that, in addition to anti-oxidative effects, LDR also prevents inflammation to protect against DN (43, 51, 55, 56). Patients with DN have poor renal function; however, most drugs used to treat DN are excreted through the kidney, which further increases the renal working load in diabetic patients. Therefore, the use of LDR as a non-invasive approach has been investigated to prevent chronic renal diseases (4, 47). In the present study, we combined LDR and FGF21 as a therapeutic strategy for the first time to explore whether simultaneously blocking the systemic and renal pathogeneses could further prevent the development of DN.

We developed a human-like type 2 diabetic model using a HFD/STZ protocol. After the model of type 2 diabetes was established successfully, the mice were exposed to LDR (50 mGy/day, every other day for 4 weeks) with and without FGF21 (1.5 mg/kg, daily for 8 weeks) based on doses optimized in our previous studies (43).
In the early-stage of DN, some abnormalities are frequently associated with enlarged kidneys. We evaluated renal hypertrophy by calculating the kidney weight to tibia length ratio. The diabetes-induced increase in this ratio was suppressed slightly by LDR and significantly by FGF21 alone and the combination treatment. In addition to renal hypertrophy, morphological analysis revealed obvious glomerular basement membrane thickening, mesangial cell proliferation, mesangial matrix expansion, glomerulosclerosis, and fibrosis in the diabetic kidneys. Diabetes also caused severe renal dysfunction, as characterized by increased UP levels and mAlb excretion. These pathological and functional diabetic changes were reduced by each single treatment strategy, and suppressed further by the combination treatment. Taken together, these findings suggest that the combination of LDR and FGF21 exerts additive renal protective effects in a model of diabetes.

Next, we assessed whether the combination treatment could be attributed to preventing both systemic and renal pathogenic changes. First, we examined blood glucose levels and plasma lipid profiles as global indicators of diabetes. Data revealed that FGF21, but not LDR, treatment lowered blood glucose levels significantly. The combination treatment exhibited a similar lowering effect on blood glucose as FGF21 treatment alone, suggesting that the hypoglycemic activity of the combination treatment could be attributed completely to FGF21. Hyperglycemia is always correlated positively with insulin resistance in patients with type 2 diabetes. As expected, FGF21 treatment improved insulin sensitivity significantly, which was enhanced further by the combination treatment. However, LDR also improved insulin sensitivity, implying that there must be other mechanisms by which LDR contributes to the improved insulin sensitivity. Strong evidence suggests that dyslipidemia is a key cause of
insulin resistance. Excessive levels of FAs and their derivatives function as signaling molecules that activate protein kinases (20). These kinases can then impair insulin signaling, finally leading to insulin resistance (39, 43). Since our animal model was characterized by obesity and insulin resistance (43), we next examined whether the different therapeutic strategies exerted beneficial effects to correct the abnormal lipid profiles in diabetic mice. Consistent with our hypothesis, obvious dyslipidemia was observed in diabetic mice, as characterized by increased plasma TG levels. TG and FFA levels were also upregulated in diabetic kidneys. Treatment with either LDR or FGF21 alone corrected the diabetes-induced abnormal lipid profiles greatly, which was enhanced further by the combination treatment. Therefore, it is likely that the additive effects of LDR and FGF21 on insulin sensitivity were due mainly to correction of the abnormal lipid profile.

Next, we assessed whether the combination strategy also afforded local renal protection at the cellular and molecular levels, in addition to the abovementioned systemic effects. Large bodies of evidence have demonstrated that oxidative stress, inflammation, and subsequent fibrosis are the main pathogeneses of diabetes in the kidney (1, 19, 50). In the current study, we confirmed that diabetes increased the levels of 3-NT and MDA significantly as markers of nitrosative and oxidative damage, and decreased the expression of multiple antioxidants and their upstream transcription factor Nrf2. Moreover, inflammatory cell infiltration and increased levels of multiple inflammatory factors such as ICAM-1, TNF-α, and PAI-1 were also observed in diabetic kidneys. Fibrosis, which reflects late-stage pathological damage, was also observed in the kidneys of diabetic mice, as determined using renal CTGF expression and Masson’s trichrome staining. Consistent with our hypothesis, all these
pathological changes were suppressed significantly by either LDR or FGF21 treatment, and
the combination treatment further protected the kidneys from injury. Therefore, the
combination of LDR and FGF21 could confer protection against renal damage.

In summary, the present study showed that exposing mice to the combination of LDR
and FGF21 exerted additive effects to further protect against type 2 diabetes induced renal
damage. These effects were exerted by blocking hyperglycemia, dyslipidemia, renal oxidative
stress, inflammation, and fibrosis. In addition, FGF21 regulates carbohydrate and lipid
metabolism without inducing hypoglycemia and cell growth. Meanwhile, since whole-body
exposure to LDR is easy to perform, non-invasive, and does not increase the kidney workload,
this combination strategy might provide a novel therapeutic approach for diabetic patients in
the near future.

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Disclosure

The authors declare they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

Author contributions

M.S., L.Y., F.Z., Y.T., P.C., X.L., L.H., H.Y., and C.Z. performed the experiments; M.S., X(M).L., Y.T., S.J., C.Z., and L.C. analyzed the data; M.S. and C.Z. prepared the figures; M.S., L.Y., F.Z., X(M).L., X(K).L, Y.T., P.C., X.L., L.H., S.J., H.Y., C.Z., and L.C. approved the final version of manuscript; M.S., X(K).L., C.Z., and L.C. conception and design of research; C.Z. interpreted the results of the experiments; M.S., C.Z., and L.C. edited and revised the manuscript. C.Z. and L.C. drafted the manuscript.
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Figure legends

Figure 1. Establishing a type 2 diabetic mouse model using the HFD/STZ. C57BL/6J mice were fed a HFD (40% of calories from fat) for 12 weeks to induce obesity. Body-weight (A), glucose tolerance (B), and insulin sensitivity (C) were examined. Three days after the injection of STZ blood glucose was measured (D). The mice were regarded as diabetic once hyperglycemia was observed (>12 mmol/L). Data are presented as means ± SEM; *p < 0.05 vs. the HFD group; #, p < 0.05 vs. the DM group. HFD, high-fat diet; DM, diabetes mellitus; GTT, glucose tolerance test; ITT, insulin tolerance test.

Figure 2. Effects of the combination treatment strategy on hyperglycemia and insulin resistance. Blood glucose levels (A) and insulin tolerance (B) were examined in each group. The data are presented as means ± SEM; n = 9 per group. *, p < 0.05 vs. the HFD group; #, p < 0.05 vs. the DM group; &, p < 0.05 vs. the LDR group; $, p < 0.05 vs. the FGF21 group. HFD, high-fat diet; DM, diabetes mellitus.

Figure 3. Effects of the combination treatment strategy on diabetes-induced histopathological changes in the kidneys of mice. Representative images of hematoxylin and eosin (A, H&E) staining, periodic acid-Schiff (B, PAS) staining, and Masson’s Trichrome staining (C, Masson) to detect renal pathological changes, glomerulosclerosis, and collagen deposition, respectively. 400× magnification. Glomerulosclerosis and collagen accumulation were examined in PAS- or Masson’s-stained slices, respectively, and quantified using Image-Pro plus 6.0 software (D, E). Renal CTGF expression was measured using western blotting (F). Renal hypertrophy was examined by assessing the change in the kidney weight to
tibia length ratio (G). Data are presented as means ± SEM; n = 9 per group. *, p < 0.05 vs. the HFD group; #, p < 0.05 vs. the DM group; &, p < 0.05 vs. the LDR group. HFD, high-fat diet; DM, diabetes mellitus.

Figure 4. Effects of the combination treatment strategy on diabetes-induced oxidative stress in the kidneys. The expression of the oxidative damage markers 3-NT (A) and renal MDA (B) were measured using western blotting and ELISA, respectively. Data are presented as means ± SEM; n = 9 per group. *, p < 0.05 vs. the HFD group; #, p < 0.05 vs. DM group; &, p < 0.05 vs. the LDR group; $, p < 0.05 vs. the FGF21 group. HFD, high-fat diet; DM, diabetes mellitus.

Figure 5. Effects of the combination treatment strategy on renal inflammation in type 2 diabetic mice. Renal tissues were collected from the different groups at the indicated times, and ICAM-1, TNF-α, and PAI-1 expression was measured using western blotting (A–C). Data are presented as means ± SEM; n = 9 per group. *, p < 0.05 vs. the HFD group; #, p < 0.05 vs. DM group; &, p < 0.05 vs. the LDR group; $, p < 0.05 vs. the FGF21 group. HFD, high-fat diet; DM, diabetes mellitus.

Figure 6. Effects of the combination treatment strategy on the expression of renal Nrf-2 and its downstream targets in type 2 diabetic mice. Renal tissues were collected from the different groups at the indicated times, and SOD-1, NQO-1, HO-1, and Nrf-2 expression was measured using western blotting (A–D). Data are presented as means ± SEM; n = 9 per group. *, p < 0.05 vs. the HFD group; #, p < 0.05 vs. the DM group; &, p < 0.05 vs. the LDR group; $, p < 0.05 vs. the FGF21 group. HFD, high-fat diet; DM, diabetes mellitus.
Table 1. The renal function of mice fed a high-fat diet with or without additional treatments

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HFD</th>
<th>DM</th>
<th>DM/LDR</th>
<th>DM/FGF21</th>
<th>DM/Com</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine protein (μg/day)</td>
<td>120.9 ± 985.4 ±</td>
<td>750.5 ±</td>
<td>664.2 ±</td>
<td>480.2 ±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.5</td>
<td>25.9 *</td>
<td>30.2 #</td>
<td>34.0 #</td>
<td>30.6 # &amp;$</td>
</tr>
<tr>
<td>Urine mAlb (μg/day)</td>
<td>17.8 ± 305.2 ±</td>
<td>184.8 ±</td>
<td>138.8 ±</td>
<td>102.5 ±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>24.7 *</td>
<td>21.2 #</td>
<td>23.1 #</td>
<td>20.3 # &amp;$</td>
</tr>
<tr>
<td>Urine creatinine (μmol/L)</td>
<td>33.8 ± 31.2 ±</td>
<td>35.5 ±</td>
<td>37.6 ±</td>
<td>38.3 ±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.0</td>
<td>1.2</td>
<td>1.5 #</td>
<td>1.4 #</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM (n = 9/group). mAlb, microalbumin; HFD, high-fat diet; DM, diabetes mellitus. *, p < 0.05 vs. the HFD group; #, p < 0.05 vs. the DM group; &, p < 0.05 vs. the LDR group; $, p < 0.05 vs. the FGF21 group.

Table 2. Plasma and renal lipid metabolic parameters in mice fed a high-fat diet with or without additional treatments

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HFD</th>
<th>DM</th>
<th>DM/LDR</th>
<th>DM/FGF21</th>
<th>DM/Com</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma TG (mmol/L)</td>
<td>1.39 ± 6.58 ±</td>
<td>4.28 ±</td>
<td>3.60 ±</td>
<td>3.06 ±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.17</td>
<td>0.70 *</td>
<td>0.72 #</td>
<td>0.65 #</td>
<td>0.48 # &amp;$</td>
</tr>
<tr>
<td>Plasma CHO (mmol/L)</td>
<td>4.41 ± 4.70 ±</td>
<td>4.58 ±</td>
<td>3.83 ±</td>
<td>3.65 ±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.47</td>
<td>0.38</td>
<td>0.34</td>
<td>0.68 #</td>
<td>0.37 #</td>
</tr>
<tr>
<td>Plasma HDL (mmol/L)</td>
<td>1.95 ± 1.65 ±</td>
<td>2.35 ±</td>
<td>2.42 ±</td>
<td>2.47 ±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.24</td>
<td>0.28 #</td>
<td>0.17 #</td>
<td>0.21 #</td>
</tr>
<tr>
<td>Plasma LDL (mmol/L)</td>
<td>0.45 ± 0.66 ±</td>
<td>0.53 ±</td>
<td>0.50 ±</td>
<td>0.35 ±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.07</td>
<td>0.16</td>
<td>0.07</td>
<td>0.08 #</td>
<td>0.07 # &amp;$</td>
</tr>
<tr>
<td>Renal TG (mg/g tissue)</td>
<td>6.17 ± 9.82 ±</td>
<td>6.20 ±</td>
<td>4.45 ±</td>
<td>3.52 ±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>1.42 *</td>
<td>1.13 #</td>
<td>1.20 #</td>
<td>1.50 # &amp;$</td>
</tr>
<tr>
<td>Renal FFA (mg/g tissue)</td>
<td>9.58 ± 16.25 ±</td>
<td>10.46 ±</td>
<td>8.20 ±</td>
<td>7.15 ±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.72</td>
<td>1.64 *</td>
<td>1.55 #</td>
<td>1.69 #</td>
<td>1.82 # &amp;$</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM (n = 9/group). HFD, high-fat diet; DM, diabetes mellitus; TG, triglycerides; CHO, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FFA, free fatty acids. *, p < 0.05 vs. the HFD group; #, p < 0.05 vs. the DM group. &, p < 0.05 vs. the LDR group; $, p < 0.05 vs. the FGF21 group.
Figure 1

A

Body Weight (g)

Con → HFD

weeks

0 2 4 6 8 10 12

0 10 20 30 40

B

Plasma Glucose (mmol/L)

IP GTT → ip glucose (1.5 g/kg)

0 30 60 90 120 min

0 10 20 30

C

IP ITT → ip Insulin (0.5 U/kg)

0 30 60 90 120 min

0 4 8 12

D

Plasma Glucose (mmol/L)

Con HFD HFD/STZ

0 4 8 12 16 20

* #
Figure 2

A

Plasma Glucose (mmol/L)

B

Plasma Glucose (mmol/L)

Area Under Curve
Figure 4

A

![Bar graph showing 3-NT and β-actin levels in different conditions: HFD, DM, DM/LDR, DM/FGF21, DM/Com.]

B

![Bar graph showing kidney MDA levels in different conditions: HFD, DM, DM/LDR, DM/FGF21, DM/Com.]

Figure 5

A

ICAM-1

β-actin

B

TNF-α

β-actin

C

PAI-1

β-actin
Figure 6

**A**

![Graph showing SOD-1 protein levels](image)

**B**

![Graph showing NQO-1 protein levels](image)

**C**

![Graph showing HO-1 protein levels](image)

**D**

![Graph showing NF-2 protein levels](image)