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Nelumbo nucifera leaves extract attenuate the pathological progression of diabetic nephropathy in high-fat diet-fed and streptozotocin-induced diabetic rats

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ABSTRACT

Diabetic nephropathy is not only a common and severe microvascular complication of diabetes mellitus but also the leading cause of renal failure. Lotus (*Nelumbo nucifera*) possesses antioxidative and anticancer properties. The present study aimed to investigate the antidiabetic and renoprotective effects of *N. nucifera* leaf extract (NLE) in a rat model of type 2 diabetic mellitus. Male Sprague–Dawley rats with type 2 diabetes induced by a high-fat diet (HFD)/streptozotocin (STZ) were treated with NLE at dosages of 0.5% and 1% (w/w) daily for 6 weeks. At the end of the experimental period, body weight, serum glucose levels, insulin levels, and kidney function were assessed. Furthermore, antioxidant enzyme and lipid peroxide levels were determined in the kidney, and histopathological examination was performed using hematoxylin and eosin staining, periodic acid Schiff staining, and Masson trichrome staining. To shed light on the molecular mechanism underlying the functioning of NLE, mouse glomerular mesangial cells (MES-13) treated with high glucose (HG, 25 mM glucose) were chosen as a model for an examination of the signal transduction pathway of NLE. The results revealed that NLE improved diabetic kidney injury by reducing blood glucose, serum creatinine, and blood urea nitrogen levels and enhanced antioxidant enzyme activities in kidney tissue. Treatment with NLE significantly reduced the malondialdehyde and 8-hydroxy-2-deoxyguanosine levels and increased serum insulin levels; expression of renal superoxide dismutase, catalase, and glutathione peroxidase activities; and glutathione content. Histological studies have also demonstrated that NLE treatment inhibited the dilation of Bowman's capsule, which confirmed its renoprotective action in diabetes. In addition, treatment with NLE and its major component quercetin 3-

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glucuronide attenuated 25 mM HG-induced suppressed nuclear factor erythroid 2-related factor 2 and antioxidant enzyme expression in MES-13 cells. Collectively, these findings indicate that NLE may have antidiabetic and renoprotective effects against HFD/STZ-induced diabetes, at least in part, through antioxidative pathways.

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1. Introduction

Hyperglycemia is the main risk factor for the development of diabetic nephropathy (DN) [1]. DN is one of the most severe complications that affects 20%–40% of all patients with type 2 diabetes mellitus (T2DM); DN develops into end-stage renal disease [2], ultimately resulting in multiorgan failure and death [3]. Substantial evidence suggests that hyperglycemia induces oxidative stress by increasing the production of reactive oxygen species (ROS) and then causing various carbohydrate metabolism changes and complications including nephropathy [4]. In DN, ROS causes oxidative stress-related renal damages, which produces marked structural and functional changes in glomerular and renal tubular cells [5]. Normally, renal cells protect themselves against ROS-induced damage through the use of various antioxidants such as enzymatic [superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)] and nonenzymatic [glutathione (GSH)] compounds. Among these antioxidants, SOD and CAT are known to be regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2) [6]. The Nrf2 transcription factor plays a key role in inhibiting oxidative stress and lipid accumulation in T2DM [6]. Recently, there has been growing evidence that the protective effects of dietary antioxidants can be explored as a potential adjuvant therapy to prevent or delay diabetic complications [7]. T2DM was induced in a well-established animal model through a high-fat diet (HFD) combined with a low dose (approximately 35 mg/kg) of streptozotocin (STZ), which mildly inhibits beta cell function, thereby developing features of DN and renal damage [8]. Therefore, treatment strategies targeting oxidative stress may effectively preserve normal renal function and halt or delay the progression of DN.

Nelumbo nucifera, generally called lotus, is an aquatic plant belonging to the family Nelumbonaceae, extensively planted in Asia and Africa [9]. Currently, leaves of the herb are gaining popularity in Asia for use as a tea drink and dietary supplement for losing body weight, reducing blood lipids, controlling blood pressure, and as an oxidant. Furthermore, lotus leaves have been primarily used to treat sunstroke, diarrhea, sweating, bleeding, and fever in traditional Chinese medicine [9]. Modern pharmacological studies have demonstrated that lotus leaves exhibit a wide range of biological activities, such as antihyperlipidemic, antidiabetic, antiobesity, antioxidant, antitumor, and hepatoprotective effects and other activities [10,11]. The most therapeutically beneficial effects of lotus are caused by or associated with its compounds. Various compositions of different chemical groups have been derived from this plant, including alkaloids, flavonoids, glycosides, and

triterpenoids. Flavonoids are the main functional components of the lotus leaves, and many of them have been identified, including quercetin, kaempferol, leucocyanidin, leucodelphinidin, catechin, isoquercitrin, and astragalin [12]. Of these, quercetin 3-glucuronide (Q3GA), also known as miquelianin, is one of the major flavonoids and an antioxidative quercetin metabolite in rats [13] and human plasma [14]. Flavonoids may play a role in many metabolic processes involved in T2DM, as shown in several in vitro and in vivo animal models and in some human studies [15]. In this context, many currently available medicines for the management of diabetes have undesirable side effects. Therefore, researchers have explored natural products with low toxicity and a hypoglycemic effect to effectively control blood glucose, prevent or reverse diabetic complications, and improve the quality of life in T2DM patients.

In our previous study, the protective effects of *N. nucifera* leaf extract (NLE) extracted from *N. nucifera* leaves against liver and breast carcinogenesis was investigated [16,17], owing to its anti-metastasis and antioxidant activities [18]. However, the protective effects of NLE on DN have not been fully elucidated. Hence, the present study aimed to evaluate the content of active components in NLE and their antioxidant activities in vivo and in vitro to assess their alleviative effects on glucose and lipid metabolic disorders in HFD/STZ-induced type 2 diabetic Sprague–Dawley (SD) rats. To evaluate the action mechanism, we hypothesized that NLE improves kidney function partly through antihyperglycemia and partly through attenuating oxidative stress in diabetic rats. In particular, we analyzed the renal functional and structural changes, serum lipid profile, glycemic status, oxidative stress parameters, and expression of the antioxidant defense enzymes in the kidneys of T2DM rats. The study results support our hypothesis, which provides new insights into the protective effects on the kidney in T2DM and its complications.

2. Materials and methods

2.1. Extraction and identification of NLE

The lotus (*N. nucifera* Gaertn) leaves was provided by a farmer in Tainan, Taiwan. NLE was extracted and purified from dry lotus leaves and identified using our previously published HPLC-UV method [19]. Briefly, fresh lotus leaves were harvested and immediately air-dried at 50 °C. The dried lotus leaves powder (200 g) was subjected to extraction using 5000 mL distilled water for 24 h at 4 °C. The residue was removed by filtration using Whatman® paper and then the

filtrate was concentrated under reduced pressure. Finally, the concentrated solution was frozen at -80°C followed by lyophilization to obtain NLE. The average yield of dry NLE powder was approximately 17.5%. The lyophilized powder was stored at -80°C until use.

2.2. Cell culture and cell viability assay

MES-13 cells (BCRC 60366; mouse glomerular mesangial cell line) were obtained from the Bioresource Collection and Research Center, Hsinchu, Taiwan. Cells were normally cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum, 5.5 mM glucose and 1% penicillin/streptomycin, unless indicated otherwise. These cell lines were at 37°C in a humidified atmosphere with 5% CO_2 . Either NLE (0–2.0 mg/mL) or Q3GA (0–50 μM) was incubated with MES-13 cells and meanwhile treated cells with or without 25 mM of glucose (high glucose, HG) for 24 h. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

2.3. Experimental design and induction of high-fat diet/streptozotocin (HFD/STZ) rat model of T2DM

Male Sprague–Dawley (SD) rats (250 ± 20 g) were supplied by LASC Corporation (Taipei, Taiwan). The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chung Shan Medical University (Approval Number: 1070). All the procedures of animal care were conducted in accordance with institutional guidelines. Rats were given seven days to acclimate to their new environment under standard laboratory conditions (a 12/12-h light/dark cycle, $22 \pm 2^{\circ}\text{C}$, $55 \pm 5\%$ humidity). All rats were provided with standard pellet diet and water *ad libitum* before any food-related treatment began. After acclimatization for 7 days, rats were fed high fat diet [20], for the initial period of 6 weeks. After 6 weeks of dietary manipulation, the HFD-fed animals received single intraperitoneal (i.p.) injection of low dose of streptozotocin (STZ, 35 mg/kg bw) as described previously [8,21]. Rats were tested for successful induction of diabetes after 7 days of STZ injection. Only those rats whose blood glucose level more than 200 mg/dL were considered to be T2DM rats and enrolled in the study. The overall experimental scheme has been depicted in Fig. 1A.

Rats were randomly divided into six groups of eight rats each, as follows:

- Group I (C): Normal control rat group were fed basal diet throughout the experiment. The experiment period was total 12 weeks.
- Group II (HFD): Rats were fed HFD throughout the experiment.
- Group III (STZ): Rats received a single i.p. injection of STZ. The animals were kept on normal pellet diet for the rest of experimental period.
- Group IV (HFD+STZ): Diabetic control rats, received HFD initially for 6 weeks followed by a single i.p. injection of STZ. The animals were kept on HFD for the rest of experimental period.

- Group V (HFD+STZ+NLE 0.5%): Diabetic rats, received NLE 0.5% (w/w) for 6 weeks post induction of diabetes. The animals were kept on HFD for the rest of experimental period.
- Group VI (HFD+STZ+NLE 1%): Diabetic rats, received NLE 1% (w/w) for 6 weeks post induction of diabetes. The animals were kept on HFD for the rest of experimental period.

At the end of the experiments, all rats were sacrificed and the kidneys and blood were harvested. Blood samples were centrifuged at $3000\times g$ for 10 min at 4°C and stored at -80°C until the various biochemical analyses. The kidneys were immediately subjected to different processing for histological, biochemical and immunoblot analyses.

2.4. Renal morphology assessment and IHC analysis

Fresh renal tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned by a microtome at 5- μm thickness. For histopathological examination, kidneys from different groups were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and Masson's trichrome (MT), according to routine staining steps that were described previously [22]. H&E stain has been used for routine histopathological observations. MT stain was used to determine the expression of collagen. PAS stain was used to evaluate the degree of glomerulosclerosis, which was defined as thickening of the basement membrane and mesangial expansion. Stained renal sections were examined by light microscopy for evaluation of histological alterations characteristics of diabetic renal disease, at least ten fields per slide were observed under a light microscope (Nikon, Tokyo, Japan). Renal damage was evaluated by a renal pathologist. Immunohistochemistry for 8-hydroxy-2-deoxyguanosine (8-OHdG) was also performed in rat kidney paraffin sections, which according to the manufacturer's instructions. Cells were in see positive expression of brown-yellow granules.

2.5. Serum biochemical measurements

Insulin and other biochemical parameters were measured at the end of study. The serum was placed in an ice-cold Eppendorf tube, centrifuged at 3000 rpm for 10 min and 200 μL of the supernatant was removed for analysis by (pro) insulin ELISA (Merckodia, 10-1124-10; detects both proinsulin and insulin), following the manufacturer's instructions. Biochemical parameters such as glucose, creatinine and blood urea nitrogen (BUN) were measured in the serum of rats using commercially available kits (Randox Laboratories, Ltd., Antrim, U.K.).

2.6. Lipid peroxidation and antioxidant enzymes assays

The tissue homogenate was used for the determination of lipid peroxidation and antioxidant enzyme. Lipid peroxidation was determined by measuring homogenate malondialdehyde (MDA) concentration, based on its reaction with thiobarbituric acid in acidic medium to form thiobarbituric acid reactive substances (TBARS). The results were expressed as MDA

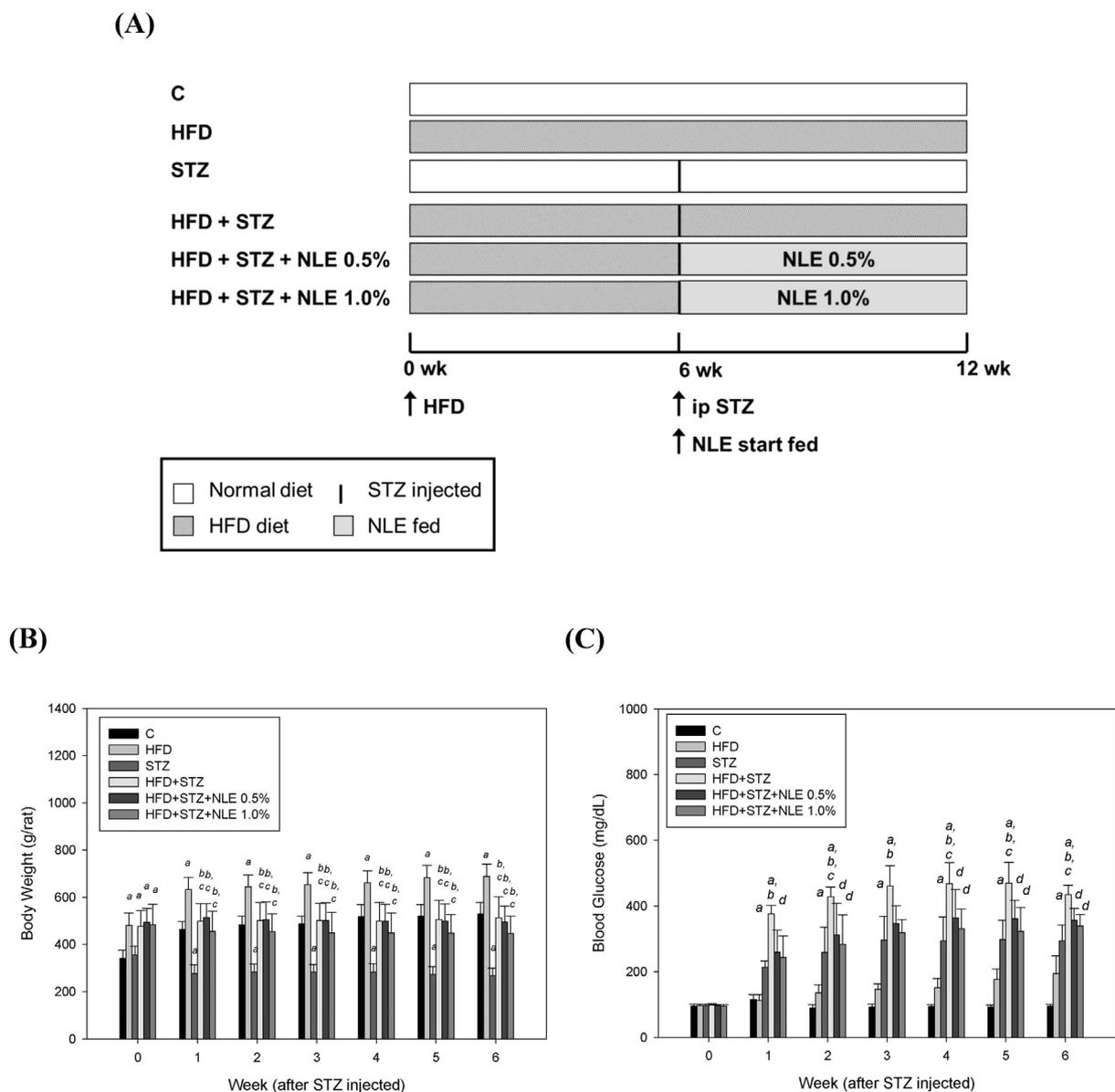


Fig. 1 – The effect of NLE on body weight and blood glucose changes in T2DM rats. (A) Schematic of group design during 16 weeks. (B) Body weight and (C) blood glucose were determined according to description in the materials and methods. After STZ injection, rats fed with a high-fat diet or a normal diet developed frank hyperglycemia at the first week (termed week 0). Abbreviations: C, normal control group; HFD, high fat diet (HFD) fed without STZ injected group; STZ, normal diet fed with STZ injected group; HFD+STZ, HFD fed with STZ injected to induced T2DM group; HFD+STZ+NLE 0.5%, HFD and 0.5% NLE fed with STZ injected group; HFD+STZ+NLE 1%, HFD and 1% NLE fed with STZ injected group. Data are shown as means ± SD (n = 8 for each group). a, p < 0.05 compared with C (control) group; b, p < 0.05 compared with HFD group; c, p < 0.05 compared with STZ group; d, p < 0.05 compared with HFD+STZ group.

equivalents using 1, 1, 3, 3 tetramethoxypropane as a standard. The absorbance of the supernatant was detected at 532 nm. The results are expressed as MDA mmol/mg protein. Antioxidant enzyme activities in the kidneys were assayed using commercial kits.

2.7. Western blot analysis

Animal protein of homogeneous kidney samples and MES-13 cell protein were lysed, quantified, and subjected to Western blot as previously described [19]. Briefly, protein samples

were separated on 10–12% SDS-PAGE and transferred onto a nitrocellulose membrane, incubated with primary antibody SOD, CAT, GPx, Nrf2 or β-actin overnight at 4 °C after blocking with non-fat milk for 1 h. Blots were washed and incubated with the appropriate secondary antibodies for 1 h at room temperature. Finally, the protein bands were detected by enhanced chemiluminescence and exposed for 1 min in a LAS-4000 Luminescent Image Analyzer (Fujifilm Corporation, Tokyo, Japan). The intensity of each band was determined through densitometry using Fujifilm Multi Gauge V2.2 software.

2.8. Detection of intracellular reactive oxygen species (ROS)

Intracellular ROS generation was detected using the DCFH-DA fluorescence assay according to the manufacturer's instructions. Briefly, the MES-13 cells were plated in 75T flask at the density of 1×10^7 cells, and exposed to appropriate concentrations of test samples. Then the cells were harvested and resuspended in dichlorofluorescein-diacetate (DCFH-DA, $1 \mu\text{M}$) buffer at 37°C for 30 min, which was analyzed by a flow cytometer. Data were acquired using a FACSCalibur flow cytometer (Becton Dickinson) with CellQuest software (Becton Dickinson).

2.9. Statistical analysis

All quantitative data were expressed as mean \pm SD for at least three analyses for each sample. Comparisons between the two groups of samples were performed using unpaired Student's *t*-test, whereas multiple comparisons of more than two groups of samples were performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Identification of NLE

High-performance liquid chromatography (HPLC) analysis of water extract of *N. nucifera* confirmed the presence of flavonoids as the major phytochemicals. On the basis of our previous study that reported the composition and content of selected flavonoids in NLE, the levels of six flavonoids in NLE were analyzed in detail using HPLC-ultraviolet (UV) [19]. In this study, the average daily consumption of feed for individual SD rats was approximately 30 g. Six phenolic compounds were identified, namely gallic acid (916.5 mg/30 g diet), catechin (1113.0 mg/30 g diet), peltatoside (532.5 mg/30 g diet), rutin (697.5 mg/30 g diet), isoquercitrin (424.5 mg/30 g diet), and Q3GA (1321.5 mg/30 g diet). Data revealed that Q3GA was the most abundant ingredient in these standards.

3.2. Effects of NLE on blood glucose level and body weight of diabetic rats

Before STZ induction, basal blood glucose levels did not differ significantly between groups. During the treatment, blood glucose level and body weight were measured once in a week in the experimental groups. As expected, a continuous body weight gain was noted in the HFD group, and body weight loss was noted in the STZ group throughout the experiment, compared with control groups (Fig. 1B). After treatment of these HFD+STZ-induced diabetic rats with a high dose (HFD+STZ+NLE1% group) of NLE for 6 weeks, the body weight was significantly lower than the weights of the HFD groups. However, the control, HFD+STZ, and HFD+STZ+NLE0.5% groups did not exhibit any significant differences in body weight at any time point; the body weights of rats in these groups were still considerably lower than those of the HFD-fed rats.

As shown in Fig. 1C, after a low-dose STZ (35 mg/kg, i.p.) injection, rats fed with an HFD or a normal diet developed frank hyperglycemia at the first week (termed week 0) and maintained high levels of blood glucose (>200 mg/dL) over the 6-week study period. By contrast, oral administration of NLE1% for 6 weeks post induction of diabetes caused a decrease in the blood glucose level in diabetic rats compared with the HFD+STZ-induced diabetic rats (339.67 ± 34.82 mg/dL vs. 434.63 ± 28.78 mg/dL). The results indicate that the animal model of T2DM is successfully established through HFD+STZ treatment can be used in future experimental studies. In addition, the plasma insulin of the diabetic rats (HFD+STZ group) was lower than that of the control rats (0.053 ± 0.01 ng/mL vs. 0.187 ± 0.01 ng/mL) (Table 1). Mildly elevated insulin levels were observed at the end of the treatment period in diabetic rats treated with NLE0.5% and NLE1% (0.067 ± 0.017 ng/mL and 0.073 ± 0.01 ng/mL). We found that a high dose of NLE increased the blood insulin levels in diabetic rats compared with the HFD+STZ group. These data suggest that NLE has an antidiabetic effect, possibly because of an increase in blood insulin levels and a significant decrease in serum glucose.

3.3. Effects of NLE on biochemical parameters in diabetic rats

The metabolic data and the monitored clinical parameters of the six groups of rats are summarized in Table 1. Table 1 outlines the effects of NLE on the nephrotoxicity markers as follows: the levels of serum urea nitrogen (BUN) and creatinine in the HFD+STZ group were significantly increased compared with the control group (BUN: 22.83 ± 5.99 mg/dL vs. 11.42 ± 1.24 mg/dL; creatinine: 1.40 ± 0.09 mg/dL vs. 0.77 ± 0.05 mg/dL). NLE-administered groups exhibited reduced serum BUN and creatinine levels (Table 1). The data indicated that NLE affects the parameters of renal function and that NLE treatment could effectively improve the renal function in T2DM rats.

3.4. Effects of NLE on renal histological changes in experimental animals

Representative histological sections of kidneys stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS), and Masson's trichrome (MT) staining are depicted in Fig. 2. Photomicrographs of renal tissue sections in the control group have a normal appearance; in other words, renal tissue exhibited a normal histology with apparent glomerulus and intact tubular architecture. The renal sections of the HFD+STZ rats showed glomerular lesions characterized by glomerular hypertrophy and increased Bowman's capsule space (arrowhead) (Fig. 2, H&E panel). In diabetic rats treated with NLE, the general structure was rescued and the Bowman's capsule space was narrowed. In addition, glycogen was assessed through PAS staining (Fig. 2, PAS panel), and MT stain was used to demonstrate collagen deposition (collagen fiber was stained blue, nuclei were stained black, and cytoplasm was stained red/pink) (Fig. 2, MT panel) in the experimental groups at the end of the study. Diabetic rats showed marked increases in PAS-stained positive areas and the amount of collagen

Table 1 – Effects of NLE on serum biochemical parameters of experimental rats.

| | C | HFD | STZ | HFD+STZ+NLE (%) | | |
|--------------------|--------------|---------------------------|---------------------------|----------------------------|-----------------------------|-----------------------------|
| | | | | 0 | 0.5 | 1 |
| Insulin (ng/mL) | 0.187 ± 0.01 | 0.107 ± 0.02 ^a | 0.062 ± 0.01 ^a | 0.053 ± 0.01 ^{ab} | 0.067 ± 0.01 ^{abd} | 0.073 ± 0.01 ^{abd} |
| BUN (mg/dL) | 11.42 ± 1.24 | 9.95 ± 0.74 | 19.48 ± 6.27 ^a | 22.83 ± 5.99 ^{ab} | 12.62 ± 2.44 ^{cd} | 12.27 ± 3.34 ^{cd} |
| Creatinine (mg/dL) | 0.77 ± 0.05 | 0.72 ± 0.04 | 0.78 ± 0.12 | 1.40 ± 0.09 ^{ab} | 0.87 ± 0.08 ^a | 0.85 ± 0.05 ^{ad} |

C, normal control group; HFD, high fat diet (HFD) fed without STZ injected group; STZ, normal diet fed with STZ injected group; HFD+STZ+NLE 0%, HFD fed with STZ injected to induced type II DM group; HFD+STZ+NLE 0.5%, HFD and 0.5% NLE fed with STZ injected group; HFD+STZ+NLE 1%, HFD and 1% NLE fed with STZ injected group. Data are shown as means ± SD (n = 8 for each group). Results were statistically analyzed with ANOVA and significantly different at $p < 0.05$ according to Duncan's multiple range test. a, $p < 0.05$ compared with C group; b, $p < 0.05$ compared with HFD group; c, $p < 0.05$ compared with STZ group; d, $p < 0.05$ compared with HFD+STZ+NLE 0% group.

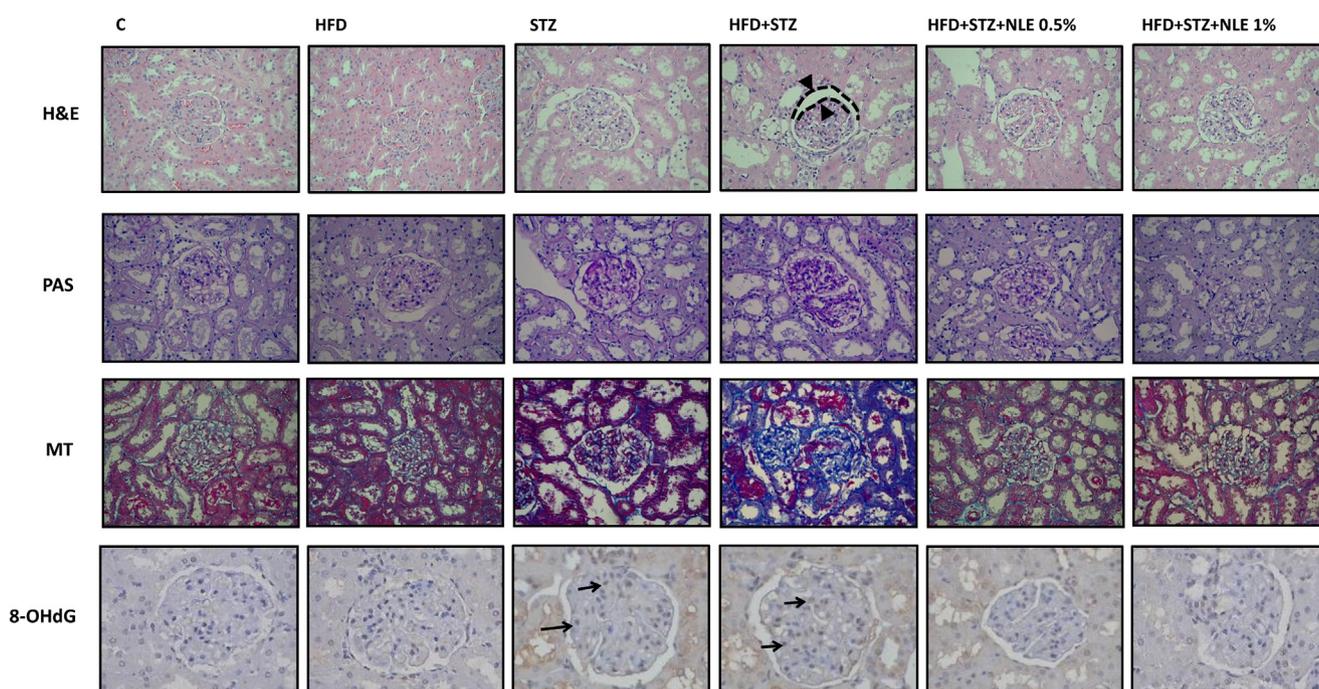


Fig. 2 – Effect of NLE on kidney histopathological picture of T2DM rats. Renal morphology and glycogen accumulation at 6 weeks evaluated by hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) staining, renal fibrosis detected by Masson's trichrome (MT) staining and 8-OHdG immunoreactivity by Immunohistochemical analysis. The H&E stained sections showed thickening of bowman's capsules (Arrowhead); PAS positive staining indicated mesangial matrix expansion; MT staining showed collagen deposition (blue color) and 8-OHdG staining assessed oxidative damage in the kidneys of T2DM rats, and representative images are provided. Black Arrows indicate the 8-OHdG-positive nuclei.

compared with normal controls. These changes in rat kidney sections were attenuated by NLE treatments. Taken together, in agreement with the blood analysis, histological examination showed that NLE treatment markedly improved the renal damage in HFD+STZ-induced diabetic rats.

3.5. Effects of NLE treatment on renal oxidative stress in T2DM rats

Oxidative stress plays a crucial role in the progression of diabetes and its complications [23]. Glucose levels were found to increase the production of free radicals, as determined by oxidative stress biomarkers such as 8-hydroxy-2-deoxyguanosine (8-OHdG) and thiobarbituric acid reactive substances (TBARS). The levels of 8-OHdG and TBARS were

measured using immunohistochemical staining and enzyme-linked immunosorbent assay (ELISA) in renal tissues. We found an increased 8-OHdG distribution pattern (black arrows) in diabetic rats compared with control (Fig. 2, 8-OHdG panel). Notably, NLE treatment could significantly reduce the positive staining compared with the HFD+STZ group. Moreover, diabetic rats exhibited a drastic increase in the renal homogenate TBARS level compared with control rats (Fig. 3A). At the end of the study, NLE-treated diabetic rats showed a marked decrease in the TBARS level compared with the HFD+STZ group.

To evaluate the oxidative renal damage following the HFD/STZ injections, the levels of the antioxidant enzyme activities of SOD, CAT, GPx, and (GSH) were measured using ELISA and immunoblot analysis. As shown in Fig. 3B–E, all of these

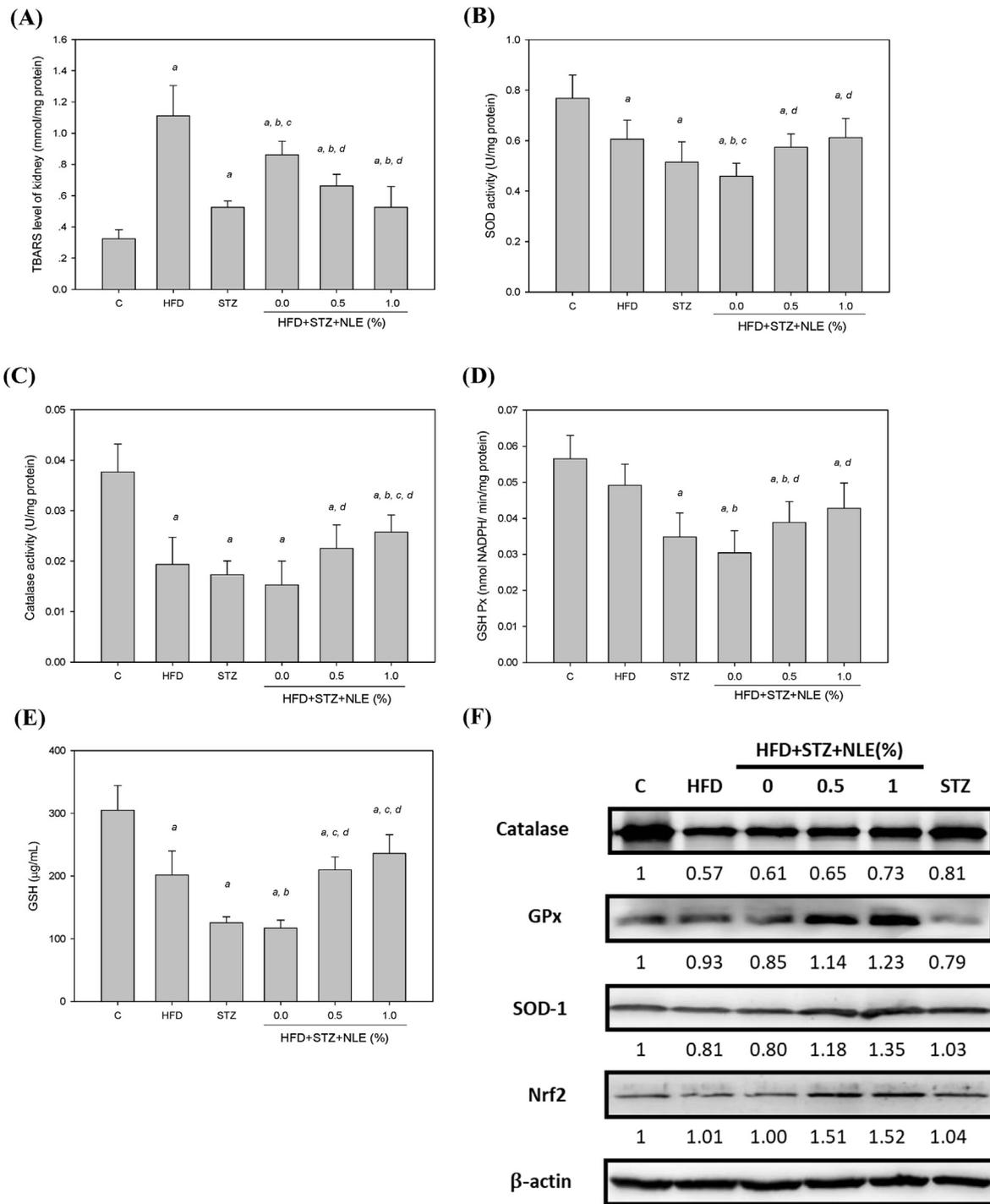


Fig. 3 – Effect of NLE on antioxidant parameters in T2DM rats. Renal tissue homogenates were used to measure antioxidant activities. (A) TBARS (MDA equivalent) concentrations. (B) Superoxide dismutase (SOD) activities. (C) Catalase activities. (D) Glutathione peroxidase (GPx) activities. (E) Glutathione (GSH) concentrations. (F) Representative Western blot bands of SOD-1, Catalase, GPx and Nrf2 in the renal tissue of rats. The relative protein expression was normalized to β -actin as a reference. The protein expression from control lysates were designated as relative induction (n-fold) compared to controls. All the experiments were performed thrice in triplicates. C, normal control group; HFD, high fat diet (HFD) fed without STZ injected group; STZ, normal diet fed with STZ injected group; HFD+STZ+NLE 0%, HFD fed with STZ injected to induced T2DM group; HFD+STZ+NLE 0.5%, HFD and 0.5% NLE fed with STZ injected group; HFD+STZ+NLE 1%, HFD and 1% NLE fed with STZ injected group. Data are shown as means \pm SD ($n = 8$ for each group). a, $p < 0.05$ compared with C group; b, $p < 0.05$ compared with HFD group; c, $p < 0.05$ compared with STZ group; d, $p < 0.05$ compared with HFD+STZ+NLE 0% group.

antioxidant activities were significantly decreased in the HFD, STZ, and HFD+STZ groups compared with the control group. As expected, the downregulation phenomena of SOD, CAT, GPx, and GSH were significantly recovered in the groups treated with 1% NLE (0.6123 ± 0.0756 U/mg, 0.0258 ± 0.00338 U/mg, 0.0428 ± 0.00703 nmol/min/mg, and 236.1195 ± 29.9163 μ g/mL, respectively) compared with the HFD+STZ group. These results were used for Western blot analysis. Consistent with Fig. 3B–E, the immunoblotting scores of SOD, CAT, GPx, and Nrf2 were significantly higher in the NLE-treated rats than in the diabetic rats (Fig. 3F). Thus, on the basis of these findings, we suggest that the underlying mechanism of NLE-induced renal protection against T2DM may involve the antioxidative pathway.

3.6. Non-cytotoxic levels of NLE and Q3GA

After confirming the protective role of NLE *in vivo*, we examined the effect of NLE in mouse glomerular mesangial cells (MES-13). In this study, MES-13 cells were treated with various concentrations of NLE and its major component Q3GA for 24 h prior to examining their viability using MTT assays. As shown in Fig. 4A and B, no obvious cytotoxic effects on cell viability were noted (>95% viability), indicating that neither NLE nor Q3GA was cytotoxic at concentrations of up to 4.0 mg/mL and 50 μ M, respectively. Thus, lower concentrations were used in subsequent experiments.

3.7. Effects of NLE treatment on high glucose (HG)-induced oxidative stress in mouse glomerular mesangial cells (MES-13)

According to Figs. 2 and 3, NLE was demonstrated to have a potent antioxidant capacity that plays a protective role against HFD/STZ-induced glomerular injury in diabetic rats. Here, we report the effect of NLE on oxidative stress in MES-13 cells measured with a dichlorofluorescein-diacetate (DCFH-DA) assay. As demonstrated by the data, a significant 4.5-fold increase in ROS production was observed in MES-13 cells treated with high glucose (25 mM glucose, HG) compared with those treated with low glucose (5.5 mM glucose, LG) (Fig. 4C). However, treatment with NLE blocked HG-induced ROS generation, similar to the results obtained from the antioxidant assays. In addition, production of intracellular ROS was suppressed by 33.24 a.u. at the 50 μ M concentration of Q3GA compared with the HG-treated cells (Fig. 4D). To confirm these *in vivo* results, we analyzed the expression of antioxidant enzymes in MES-13 cells. Consistently, protein levels of cell lysate SOD, CAT, GPx, and Nrf2 were also upregulated in NLE- or Q3GA-treated MES-13 cells compared with HG-exposed cells (Fig. 4E and F). Therefore, this evidence suggests that NLE may prevent the formation of ROS through antioxidant enzyme expression, which could be attributed to its Q3GA and phenolic content.

4. Discussion

In this study, we administered different doses of NLE to SD rats to determine its antidiabetic and renoprotective effects.

At the end of the experiment, NLE not only reduced the blood glucose, body weight, and the markers of renal functions but also improved the renal histopathological damage, thus preventing the progression of DN (Figs. 1 and 2). NLE treatments enhanced antioxidant capacity through induction of SOD, CAT, and GPx activities and GSH levels *in vivo* (Fig. 3). As a result, NLE application showed a tendency to improve symptoms or reverse disease damage despite lack of significant differences in body weight, blood glucose as well as kidney and antioxidant parameters between the NLE treated 0.5% and 1% group. Briefly, the effects of ingesting 1% of NLE are more potent than ingesting 0.5% of NLE. These changes indicated that NLE had desirable protective effects on the renal structure and physiological functions of DN rats. Further, the underlying mechanism of HG-induced injury prevention by NLE is that NLE can abrogate HG-induced oxidative stress and induce the expression of Nrf2 in MES-13 cells (Fig. 4). In addition, based on our previous reports it is known that NLE has hepatoprotective potential in different experimental animal models. For example, the three studies found that NLE (0.5–2%) possesses antioxidant and free radical scavenging activities and has a protective effect against diethylnitrosamine (DEN)-induced hepatic damage [16], high fat diet-induced hepatic injuries [24] and alcohol-induced steatohepatitis [25]. Moreover, Ho et al. indicated that there was no atherosclerotic lesion in the thoracic aorta and intima/media area ratio was no difference between untreated control rats and NLE (1%) alone treated rats [18]. Likewise, Lee et al. found that New Zealand white rabbits fed with basal diet (group I) and 1% NLE only (as toxicity group; group II) in daily diet was less atherosclerotic lesion development [26]. In animal models, we select a safe dose for appropriate daily supplements is 0.5–1% (based on administration with food), which was established according to the findings of these studies [16,18,24–26]. Thus, it is reasonable to support the notion that NLE did not produce any toxic effect in the animals' tissues, and we report here for the first time that NLE prevents DN.

High fat fed-low single dose of STZ model was used in this study and the model of T2DM was induced as described previously [8], with minor modifications. According to Srinivasan et al., prolonged exposure of high-fat diet leads to insulin resistance, and the development of diabetes occurs only in insulin-resistant HFD-fed rats following low dose STZ, because the HFD-fed rats are already mildly hyperglycemic due to insulin resistance [8]. Now, a growing number of animal studies demonstrate that a low dose of STZ combined with HFD was considered to represent the pathophysiological state of T2DM [8,21,27–29]. On the other hand, the cardinal feature of T2DM is insulin resistance. Researchers have identified that the insulin receptor substrate-1 (IRS-1)/phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB or Akt) insulin-signaling cascade is closely associated with insulin resistance-associated diseases, including diabetes and obesity [30]. Moreover, it has been reported that serine 307 phosphorylation of IRS-1 was increased and serine 473 phosphorylation of Akt was decreased in T2DM mice [31,32]. It is likely that serine phosphorylation of IRS-1 and Akt are associated with the development of insulin resistance. Therefore, based on the results of our unpublished data, NLE supplementation might improve insulin resistance by reducing the phosphorylation of

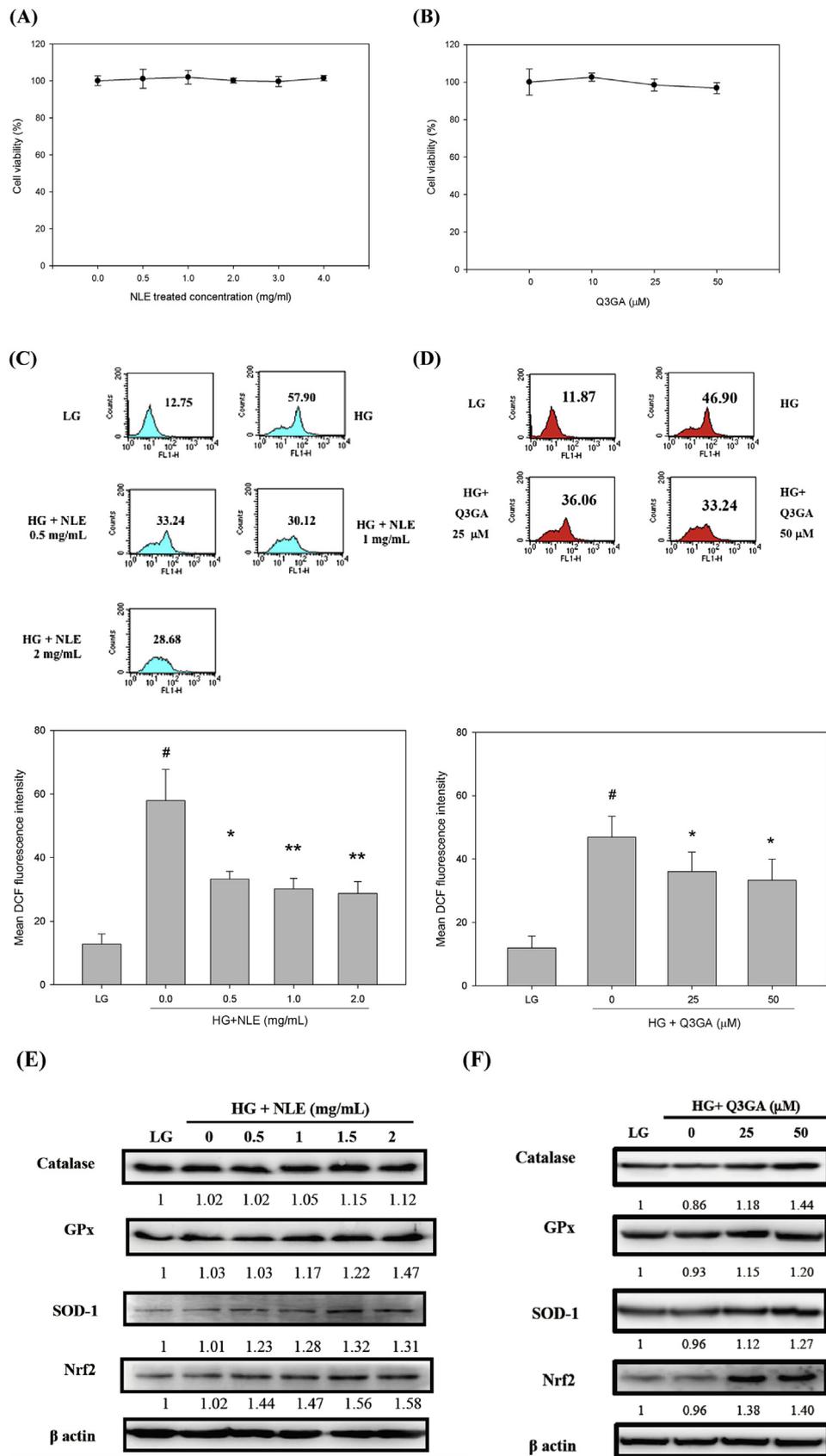


Fig. 4 – Effects of NLE and Q3GA on oxidative stress-induced cell injury and the expression of various proteins in high glucose-exposed MES-13 cells. MES-13 cells were treated with indicated concentration of NLE or Q3GA for 24 h. (A and B) Cell viability values were measured using an MTT assay. In addition, MES-13 cells were treated with various concentrations of

IRS-1 Ser307 and elevating the phosphorylation of Akt Ser473. Taken together, there is no doubt that the rodent model of HFD feeding with low-dose STZ injection is suitable for studying T2DM and its complications.

Some studies have noted that the insulin concentration in T2DM induced by combining an HFD with STZ is lower than that of a normal group [8,33–35]. For example, Srinivasan et al. indicated that injection of STZ (35 mg/kg, i.p.) after 2 weeks of dietary manipulation with HFD produced a significant reduction in plasma insulin levels (217.68 ± 26.67 pmol/L), compared with the levels of normal pellet diet-fed rats (262.00 ± 23.52 pmol/L) [8]. Mu et al. reported that the plasma insulin levels of HFD/STZ mice were significantly lower than those of normal diet-fed control mice (0.56 ± 0.05 ng/mL vs. 0.90 ± 0.09 ng/mL), whereas blood glucose levels were high [33]. Moreover, fasting blood insulin was shown to remarkably decline in the HFD-STZ group relative to the control group (9.17 ± 1.34 mU/L vs. 11.03 ± 1.68 mU/L) [34]. According to the observations, HFD combined with a low dose of STZ might induce T2DM with severe diabetic complications, which present not only insulin resistance but also insulin deficiency. Our results are in agreement with those of published studies that demonstrated that the pathological and physiological changes induced by STZ/HFD in animals may more suitably mimic those seen in human T2DM [8] and subsequently nephropathy [35]. Nevertheless, unlike our observation, a study by Peng et al. proved that insulin concentration in T2DM induced by HFD and intraperitoneal injection of low-dose STZ (35 mg/kg) was higher than that of the normal group [36]. Similarly, HFD/STZ rat models with elevated blood glucose levels coexisted with either lower, higher, or the same levels of insulin relative to lean controls [29]. Additionally, Sharma et al. reported that a single intravenous injection of STZ (50 mg/kg BW) produced a mild destruction of β cells, further reducing the amount of insulin secreted, and finally leading to symptoms of hyperglycemia [37]. Conversely, administration of the aqueous extract from *N. nucifera* leaves (200 μ g/mL) prevented body weight loss, reduced hyperglycemia, NO production, blood BUN and creatinine as well as increased insulin secretion in STZ-induced diabetic rats. Using another animal model, Huang and others demonstrated that HFD-fed mice exhibited glucose intolerance and insulin resistance (or low insulin sensitivity), as well as this condition could be significantly reversed by oral administration of NNE (the methanolic extract of *N. nucifera* leaf; 100 mg/kg) [11]. Also, both NNE and its active constituent catechin can reduce the blood sugar and stimulate the insulin secretion through a Ca^{2+} -activated PKC-regulated ERK1/2 signaling pathway in β cells (HIT-T15). Collectively, the discrepancy in results between our study and previous studies may be attributed to differences in

concentrations and times of exposure to lotus leaves or to varied origins, growing conditions, and extraction procedures of lotus leaves. Various animal models (with varying animal species, feeding period, and HFD composition) used in the experiments may also be a reason for these differences. According to our review of relevant literature, a novel finding from this study is that Q3GA-rich lotus leaf extract produced a significant antidiabetic efficacy (including delay or prevent diabetes-related kidney disease) via Nrf2-mediated antioxidant signaling pathway using T2DM rat model induced by a high-fat diet combined with a low-dose streptozotocin injection. On the other hand, one previous study reported that treatment of rats with berberine slightly decreased body weight gain but with no significant difference compared with the HFD-STZ group [34]. This is consistent with our finding that insulin deficiency delays body weight gain in the NLE treatment group (Fig. 1B and Table 1). However, many researchers used the HFD-STZ model and showed a significant gain or loss in body weight after STZ injection [29]. This can be explained by dehydration, decreased glucose metabolism [38], and excessive protein and fat catabolism [39], which ultimately led to muscle wasting [40]. Besides, administration of NLE caused a decrease in serum glucose levels and body weight in diabetic rats, but they were not restored to near-normal status. Despite this finding, we noted marked beneficial improvement in these rats. From the results, we assume that the antidiabetic effects of NLE are at least partially independent of insulin and probably occur through the inhibition of ROS production.

Evidence suggests that the pathogenesis of DN is triggered by several factors, including hyperglycemia, oxidative stress, dyslipidemia, advanced glycation end products (AGEs), and others [41], which play an important role in experimental animals as well as in diabetic patients [42]. Among them, hyperglycemia increases oxidative stress and promotes lipid peroxidation and loss of function of different cell types such as renal cells [43]. Nrf2-mediated protection against renal damage through reduction of free radicals has been reported in STZ-induced DN models [44]. As shown in Fig. 4C and D, ROS was observed to be accumulated after exposure to HG, whereas cells treated with either HG+NLE or HG+Q3GA effectively inhibited HG-induced ROS generation. Furthermore, as shown in Fig. 4E and F, expression of antioxidant enzymes SOD, CAT, GPx and Nrf2 were determined by Western blotting. Cells treated with HG+NLE or HG+Q3GA had an upregulation of SOD, CAT, GPx and Nrf2; however, we were not able to detect significant changes in protein expression of the enzymes in HG. Even so, there are numerous studies showing that the results were similar to our present findings. For instance, Piwkowska et al. found that long-term (3 and 5

NLE, Q3GA and incubated with low glucose concentrations (LG; final concentration, 5.5 mM) and HG (final concentration, 25 mM) for 24 h, respectively. (C and D) Flow cytometry analysis of the intracellular ROS. Intracellular ROS levels were measured using DCF fluorescence. The bottom panel shows statistical analysis of the flow cytometry data. (E and F) Representative Western blot bands of SOD-1, Catalase, GPx and Nrf2 in MES-13 cells. The relative protein expression was normalized to β -actin as a reference. The protein expression from control lysates were designated as relative induction (n-fold) compared to controls. Each data value is given as means \pm SD for three independent experiments. The values represent the means \pm SD of triplicate experiments. * $p < 0.05$, ** $p < 0.01$ vs. HG-treated group; # $p < 0.05$ vs. LG-treated group. Abbreviations: HG, High glucose; LG, Low glucose.

days) exposure of mouse podocytes to high glucose concentrations (30 mM) caused oxidative stress, but failed to alter SOD-1, SOD-2, SOD-3 and GPx-1 protein expressions [45]. They suggested that allosteric regulation of SOD activity in HG, probably due to compensation the increased NAD(P)H oxidase-dependent O_2^- production. In addition, Nrf2 is a transcription factor that activates antioxidant genes, and it is expressed in many different tissues including the kidney. Another report showed that the HG (30 mM) treatment increased Nrf2 expression in mouse mesangial cells and renal glutathione peroxidase (GPX) mRNA levels were increased in the OLTF (diabetic) group compared with the LETO group [46]. Tan and others demonstrated that cardiac Nrf2 expression was slightly upregulated at early stage of diabetic heart but significantly downregulated at the late stage in diabetic hearts [47,48]. In the study by Yoh et al., STZ-injection to Nrf2 knockout mice did not develop renal hyperfiltration at 2 weeks, which was seen in the STZ-treated wild-type mice, but renal function gradually deteriorated over the 10-week observation period [49]. Taken together, this phenomenon could be explained that Nrf2 has a compensatory effect for kidney protection at the early stage of diabetes; when antioxidant activity is not further impaired at the early stage of diabetes, leading to an increase (or at least no decline) in Nrf2 expression. In other words, the Nrf2 was presumably degraded in long-term conditions or chronic diseases, and it cannot regulate the expression of its downstream target genes and ultimately aggravate DN. Further works are necessary to clarify this issue.

Plant-based medicines play a vital role in the development of novel therapeutic agents. Lotus leaves have a long history of use in traditional Chinese medicine, and it or other natural products have been reported by other researchers on the antidiabetic activity [50]. Because the results of this study confirmed that NLE exhibited an antioxidant effect in vivo, effects of NLE on antioxidant enzymes and oxidative stress biomarkers in MES-13 cells were studied to investigate whether an antioxidant effect operated in vitro. Notably, a systematic review and meta-analysis of clinical studies that focused on supplementation of dietary sources of antioxidants alone or in combination suggested that such a strategy may also improve renal damage in patients' diabetic kidney disease [51]. Thus, we speculate that NLE protects against DN through antioxidant mechanisms. A recent study on rodents demonstrated that aqueous extract of lotus leaves significantly reduced visceral fat mass and ameliorated insulin resistance, which may be associated with PPAR γ 2 and GLUT4 expression in HFD-induced obese mice [52]. In addition to antidiabetic activity, several studies have verified that lotus leaves play a beneficial role in the prevention and treatment of various diseases owing to their diverse biological activities and pharmaceutical properties. For instance, Park and his colleagues reported that *Nelumbo* leaf extracts exerted strong anti-inflammatory effects, which attenuate LPS-activated macrophage responses through the inhibition of NF- κ B activation, and two major constituents of these extracts were catechin and quercetin [53]. Lotus leaf extracts contain multiple bioactive substances, particularly flavonoids, which exhibit strong antioxidant activities because of their ability to reduce the formation of free radicals and to scavenge free

radicals [54]. On the other hand, Q3GA is a type of natural flavonoid and a metabolite of quercetin [13]. Q3GA was found to be the predominant constituent of lotus leaf extracts [55]. Research has revealed that Q3GA exhibits antioxidant effects in vitro and in human plasma [13,14]. Because one of the major constituents in NLEs is Q3GA, this study examined the effect of Q3GA compared with that of NLE. In this article, we assumed that lotus leaves can prevent and even improve preclinical pathological changes to control disease progression in T2DM. These results suggest that NLE exerts beneficial effects in diabetes by improving insulin sensitivity, increasing insulin production, and reducing the amount of glucose in rats. A substantial body of scientific evidence supports the suppressive role of lotus leaves in the treatment or prevention of DM and even a positive role on health.

5. Conclusions

These findings indicate that NLE exerted anti-diabetic and renal protective properties due to its high content of total flavonoids (including Q3GA). From the aforementioned evidence, we conclude that NLE treatment may be a promising option in ameliorating diabetes and its complications, specifically DN. However, the effectiveness of NLE improved diabetic complications requires further clinical validation.

Competing financial interests

The authors declare that there are no conflicts of interest.

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Abbreviations

| | |
|--------|---|
| NLE | <i>Nelumbo nucifera</i> leaf extract |
| HFD | high-fat diet |
| STZ | streptozotocin |
| HG | high glucose |
| 8-OHdG | 8-hydroxy-2-deoxyguanosine |
| Q3GA | quercetin 3-glucuronide |
| Nrf2 | nuclear factor erythroid 2-related factor 2 |
| T2DM | type 2 diabetes mellitus |
| DN | diabetic nephropathy |
| SD | Sprague–Dawley |
| GSH | reduced glutathione |
| SOD | superoxide dismutase |
| CAT | catalase |
| GPx | glutathione peroxidase |
| H&E | hematoxylin and eosin |
| PAS | periodic acid Schiff base |
| MT | Masson's trichrome |
| IHC | immunohistochemistry |

| | |
|---------|--|
| MDA | malondialdehyde |
| ROS | reactive oxygen species |
| MTT | 3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium |
| IACUC | Institutional Animal Care and Use Committee |
| TBARS | thiobarbituric acid reactive substances |
| DCFH-DA | dichloro-dihydro-fluorescein diacetate |
| AGEs | advanced glycation end products |
| IRS-1 | insulin receptor substrate-1 |
| PI3K | phosphoinositide 3-kinase |
| PKB | protein kinase B |

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