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EXPERIMENTAL STUDY

Nelumbo Nucifera leaf extract attenuated pancreatic β-cells toxicity induced by interleukin-1β and interferon-γ, and increased insulin secretion of pancreatic β-cells in streptozotocin-induced diabetic rats

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Abstract

OBJECTIVE: To evaluate the effect of Nelumbo Nucifera leaf water extract (NNLE) on insulinoma (RIN) cells induced by interleukin-1β (IL-1β) and interferon-g (IFN-γ), and injured pancreatic β-cells induced by Streptozotocin (STZ) in rats.

METHODS: The anti-oxidative effects of NNLE were assessed using 1,1-diphenyl-2-picryl hydrazyl (DPPH) and nitric oxide (NO) scavenging assays. The inhibitory effect of NNLE on α-glucosidase and DPP (dipeptidyl peptidase)-IV was measured *in vitro*. Pancreatic β-cell protective and insulin secretory effects were assessed, using IL-1β and IFN-γ-induced rat RIN cells. STZ-induced diabetic rats were treated with 50, 100, and 400 mg/kg NNLE for 4 weeks. The effects of NNLE on blood glucose (BG), body weight (BW), and lipid profiles were measured.

RESULTS: NNLE inhibited DPPH, NO, α-glucosidase, and DPP-Ⅳ which were directly linked to the function of β-cells. Furthermore, NNLE protected RIN cells from toxicity induced by IL-1β and IFN-γ, decreased NO production, and increased insulin secretion. NNLE caused a significant reduction in blood glucose, triglyceride (TG), total cholesterol (TC), blood urea nitrogen (BUN), and creatinine in STZ-induced diabetic rats. Furthermore, it significantly decreased BW loss in STZ-induced diabetic rats.

CONCLUSION: Our results suggest that NNLE reduced the toxicity in insulinoma cells and increased insulin secretion in pancreatic β-cells in STZ-induced diabetic rats.

Key words: Nelumbo Nucifera; Diabetes mellitus; Insulin; Interleukin-1beta; Interferon-gamma; Insulin-secreting cells; Streptozotocin; Glucose

INTRODUCTION

Diabetes is caused by impaired insulin production and / or decreased tissue response to the insulin. The number of people with diabetes are increasing worldwide; About 366 million people have diabetes, and 552 million people are expected to have diabetes in 2030.[1] According to the International Diabetes Federation, every

six second a person dies from diabetes. In diabetes, insulin secreting pancreatic β-cells are injured by the selective destruction of the islets of Langerhans.[2] Infiltrated immune cells, in and around the islets of Langerhans, play an important role during the early stage of the pathogenesis of insulin dependent diabetes mellitus (IDDM).[3] Cytokines such as interleukin-1β (IL-1β) and interferon-γ (IFN-γ) are the effector molecules that play a central role during the initial destruction of pancreatic β cells; the combination of IL-1β and IFN-γ upregulates an inducible form of nitric oxide synthase expression, and subsequently NO production, which impairs insulin secretion, causing diabetes.[4] Recently, it has been reported that the protection of pancreatic β-cells play a major role in glucose homeostasis by preserving, expanding, and improving their function.[5] Moreover, recent diabetic research has been focused on how to protect and improve functional β- cells to regulate glucose homeostasis in diabetes.[6] Therefore, there is an urgent need for the development of β- cell protective and safe hypoglycemic agents that could target the intimate mechanisms of beta cell damage and change the anti-diabetic therapy scenario in near future.

Several medicinal plants with high flavonoid and polyphenol contents are reported to have not only blood glucose lowering effects, but also pancreatic β-cell protective effects.[7] Nelumbo Nucifera, commonly named lotus, is an aquatic plant belonging to the family Nelumbonacea, widely distributed in China, Japan, and India. Nelumbo Nucifera is an agricultural crop and is cultivated for food and drink for thousand of years in South Korea. Every part of the lotus, including leaves, flowers, seeds, and rhizomes, have been reported to have several medicinal values;[8] many Korean food recipes contain lotus as a healthy ingredient. Furthermore, tea, noodles, juice, etc., prepared from the lotus, have gained popularity in South Korea. Recently, Huang and his colleagues reported that 100% methanol extract of the lotus leaves reversed the glucose intolerance in high-fat-diet-induced obese mice.[9] Similarly, Liu *et al* [10] reported the hypolipidemic and α-glucosidase inhibitory effects of the total flavonoids from Nelumbo Nucifera leaves. It has been reported that the water extract of Nelumbo Nucifera leaf contains 27.2% total flavonoids and 9.3% of total phenolic acids.[11] However, to the best of our knowledge, no one has reported on its effect on pancreatic β-cells against cytokine mediated toxicity, and biochemical parameters in pancreatic β-cells-injured diabetic rats. Therefore, in this study, we hypothesized to find whether the water extract of Nelumbo Nucifera leaves could protect pancreatic beta cells in insulinoma (RIN) induced by IL-1β and IFN-γ in rats, and increase the insulin secretion in pancreatic beta cells in streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Cell culture and reagents

RIN cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and grown at 37 ℃ under a humidified 5% CO_2 atmosphere in RPMI 1640 medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) 2 mM glutamine, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 2.5 μg/mL of amphotericin B. IL-1β and IFN-γ were purchased from R & D Systems (Minneapolis, MN, USA), and rat insulin ELISA kit was purchased from Mercodia Developing Diagnostics (Mercodia, Uppsala, Sweden). 2, 2-diphenyl-1- picrylhydrazyl (DPPH), sodium nitropruside (SNP), rat intestinal acetone powder, p-Nitrophenyl α-D-gluco-pyranoside, and streptozotocin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Substrate for DPP-IV enzyme, H-Gly-Pro-AMC, was purchased from AnaSpec Inc. USA. All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise specified.

Preparation of Nelumbo Nucifera leaf extract (NNLE)

Nelumbo Nucifera leaves (2 kg) were dried and extracted with 4000 mL distilled water for 24 h at room temperature. The extraction was repeated three times. The residue was removed by filtration and the filtrate was evaporated followed by freeze drying. The yield of the extract was about 12% of the starting material. The concentration of total phenolic acids and the total flavonoid content in the NNLE was measured as described previously.[11]

Measurement of DPPH, NO, DPP-IV, and α-glucosidase

DPPH, NO, DPP-IV, and alpha glucosidase scavenging activities were measured as described previously.[12,13]

Cell viability, NO production, and insulin secretion

Cell viability, NO production, and insulin secretion were measured as described previously.[14]

Experimental animals and induction of diabetes

Six weeks old adult male albino Wistar rats were purchased from the Central Lab Animal Inc. (Seoul, Korea). They were kept at standard living conditions (Room temperature of 25 ℃, 45%-50% relative humidity and 12 h dark/light cycle) in the Animal Research Center of Mokpo National University. All the animals were provided with standard pellet diet and water ad libitum. The rats were acclimatized to the laboratory conditions for 1 week prior to the commencement of the experiment. Procedures involving animal care were conducted in conformity with the institutional guidelines of Mokpo National University, South Korea. Diabetes was induced in overnight-fasted rats by a single intravenous injection of STZ (50 mg/kg BW),

freshly dissolved in citrate buffer (pH 4.5). Normal animals were injected only citrate buffer. Rats were tested for successful induction of diabetes, after 72 h after STZ induction, by measuring the fasting blood glucose level. Only those rats whose blood glucose level > 250 mg/dL were enrolled in the study.

Experimental design

After successful induction of diabetes, rats were divided into five groups of 6 rats in each group. Rats injected with only citrate buffer served as the normal group. Rats injected with STZ served as the diabetic group. Control groups were administered only normal saline. NNLE, at three different doses, was administered orally using a gastric sonde for 4 weeks. NNLE50 group was fed with 50 mg/kg BW NNLE; NNLE200 group was fed with 200 mg/kg BW NNLE; and NNLE400 was fed with 400 mg/kg BW NNLE. Body weight, diet intake and water intake were measured twice a week during the experimental period of 4 weeks. The rats had free access to food and water ad libitum. At the end of the experiments, all rats were fasted overnight and killed by anesthetizing them with ether, and blood was collected from the abdominal artery for the various biochemical analyses. Kidneys were removed surgically, and their weight was measured. Urine volume was calculated one day before fasting for 24 h.

Measurement of blood glucose level

Blood was collected from the tail vein of overnight (10-12 h)-fasted rats in each week for the determination of blood glucose, using a blood glucose test meter (GlucoDr, Almedicus, Korea).

Measurement of advanced glycosylation end products (AGEs)

Serum (100 μL) was mixed with 100 μL of 0.6% SDS/10 mM Tris-HCl (pH 7.4) and 5 μL of 2M $NaBH_4$/50 mM NaOH in a Eppendorf tube and heated at 100 ℃ for 10 minutes on a heating block. After heating, 800 μL of 100 mM PBS (pH 7.2) was added to the reaction mixture and fluorescent reaction products were assayed by Victor[3] Multilabel Counter (PerkinElmer, Boston, MA, USA) at 370 nm excitation and 440 nm emission.

Biochemical analysis

The collected blood was immediately centrifuged at 3000 r/m for 20 min at 4 ℃. The levels of serum triglyceride (TG), total cholesterol (TC), BUN, and creatinine were measured spectrophotometrically, using commercially available kits (AsanPharm, Seoul, Korea).

Statistical analysis

The SPSS software (SPSS, Inc., Chicago, IL, USA) was used for statistical data analyses. All data are presented as mean ± standard deviation. Analysis of variance was used to assess the differences between normal and control groups. *P*-values < 0.05 were statistically considered to be significant.

RESULTS

Components of NNLE

NNLE powder was prepared as described under materials and methods. The spectrophotometric analysis of NNLE showed the presence of gallic acid, rutin, quercetin, catechin, epicatechin, and epigallocatechingallate. The total phenolic acid and total flavonoid content were found to be approximately 10% and 28.1%, respectively. We assume that flavonoids and polyphenols may be the main components in NNLE.

Effect of NNLE on DPPH and NO scavenging activities

Figure 1A depicts the dose response curve of DPPH and NO free radical scavenging activities of NNLE. 50 and 100 μg/mL NNLE inhibited DPPH radical formation by 61.6% and 69.9%. Similarly, 50 and 100 μg/mL NNLE inhibited NO radical formation by 53.6% and 57.7%.

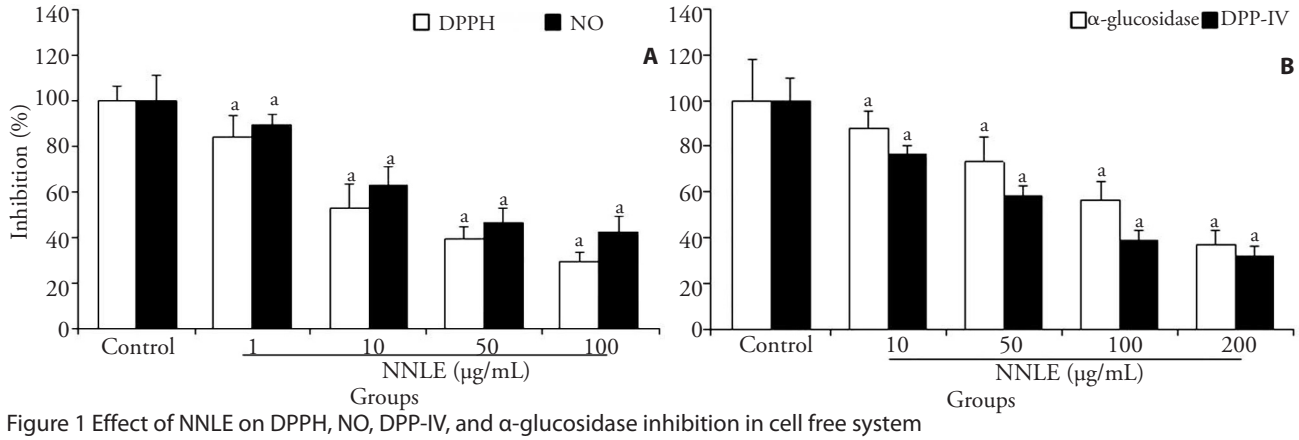

Figure 1 Effect of NNLE on DPPH, NO, DPP-IV, and α-glucosidase inhibition in cell free system

A: DPPH and NO inhibition at 1, 10, 50, and 100 μg/mL dose of NNLE. B: DPP-IV and α-glucosidase inhibition at 10, 50, 100, and 200 μg/mL dose of NNLE. Control: NNLE untreated group. NNLE: Nelumbo Nucifera leaf extract; DPPH: 2, 2-diphenyl-1- picrylhydrazyl; NO: nitric oxide; DPP-IV: dipeptidyl peptidase -IV. Results are expressed as mean ± standard deviation of percent inhibition of three independent experiments. [a]*P* < 0.05, versus untreated control group.

Effect of NNLE on α-glucosidase and DPP-IV inhibition

As shown in Figure 1B, NNLE inhibited α-glucosidase and DPP-IV activity in a dose dependent manner. The addition of 50 and 100 μg/mL NNLE inhibited α-glucosidase by 43.9% and 63.4%. Similarly, 50 and 100 μg/mL NNLE inhibited DPP-IV by 61.1% and 68.0%, respectively.

Effect of NNLE on IL-1β and IFN-γ induced cell death and NO formation in RIN cells

RIN cells were treated with various concentrations of NNLE to assess its cytotoxicity. Then, non-toxic doses of NNLE (50, 100, and 200 μg/mL) were used for further experiments. Figure 2 depicts that the combination of IL-1β and IFN-γ decreased cell viability and increased NO production. However, the addition of 100 and 200 μg/mL NNLE increased cell viability by 27.2% and 55.4%, and decreased NO production by 22.8% and 48.0%, respectively.

Effect of NNLE on the RIN cells induced by IL-1β and IFN-γ

IL-1β and IFN-γ strongly decreased insulin secretion from 44.23 to 6.88 ng/mL, that is by 84.4% (Figure 3). However, the addition of 100 and 200 μg/mL NNLE strongly increased insulin secretion from a baseline of 6.88 ng/mL to 16.43 and 23.96 ng/mL, that is by 58% and 71.2 %, respectively.

Effect of NNLE on pancreatic β-cells in STZ-induced diabetic rats

Before STZ-induction, basal blood glucose levels did not differ significantly between groups. But, after 2 weeks of STZ-induction, NNLE200 and NNLE400 decreased blood glucose level significantly (Figure 4). In the final week of the experiment, all three doses of NNLE administered rats showed decreased blood glucose level, significantly. NNLE400 decreased blood glucose level by 23.6% in the final week of the experiment.

Effect of NNLE on body weight in STZ-induced diabetic rats

There is a continuous body weight gain in normal group, and body weight loss in STZ-induced diabetic groups throughout the experiment. But, NNLE administered groups prevented body weight lost throughout the experiment, compared with control groups. NNLE400 showed 27 g gain on body weight, compared with control groups, at the end of the experiment. Furthermore, kidney weight and urine volume are significantly increased in diabetic control group, compared with normal group. But, NNLE400 groups showed significantly decreased kidney weight and urine volume (Table 1).

Effect of NNLE on serum TG, TC, BUN, AGEs, and creatinine levels

Serum TG, TC, BUN, and creatinine are increased in STZ-induced diabetic rats, compared with normal group (Table 1). NNLE significantly decreased serum TG and TC in a dose dependent manner. NNLE400 decreased serum TG and TC by 28.8% and 19.4%, respectively, compared with the control group. NNLE administered groups showed decreased serum BUN and creatinine. However, only NNLE400 group showed significant decrease in serum BUN and creatinine, compared with the control group. Similarly, NNLE400 goup showed decreased serum AGEs level by 23.6%, compared with the control group (Figure 5).

DISCUSSION

Pancreatic β-cells play a central role in glucose homeostasis, regulating different biochemical parameters.[5] Thus, protecting pancreatic β-cells, to maintain insulin secretion and glucose metabolism, is the ultimate goal of anti-diabetic therapy. In the present study, we investigated the β cells protective, and the hypoglycemic effect of NNLE, using pancreatic β-cells and STZ-induced diabetic rats. We found that NNLE pro-

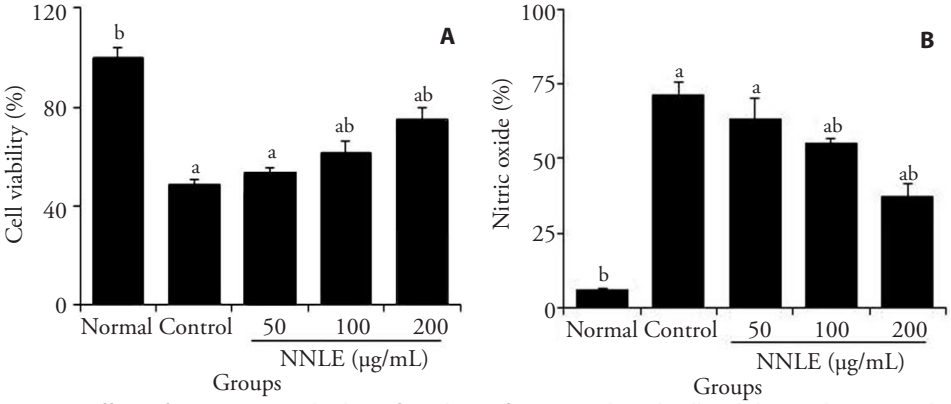

Figure 2 Effect of NNLE on interleukin-1β and interferon-γ-induced cell viability and nitric oxide productionin in insulinoma cells
A: cell viability in cytokine treated cells at 50, 100, and 200 μg/mL dose of NNLE. B: nitric oxide production in cytokine treated cells at 50, 100, and 200 μg/mL dose of NNLE. Insulinoma cells (2×10^6) were pretreated with the indicated concentrations of NNLE for 3 h, followed by stimulation with IL-1β (2 ng/mL) and IFN-γ (100 U/mL) for 48 h. Normal: untreated cells; Control: cytokine treated cells; NNLE (μg/mL): cytokine and NNLE treated cells; IL-1β: interleukin-1β; IFN-γ: interferon-g. Each value represents the mean ± standard deviation of three independent experiments. $^aP < 0.05$, versus normal group and $^bP < 0.05$, versus control group.

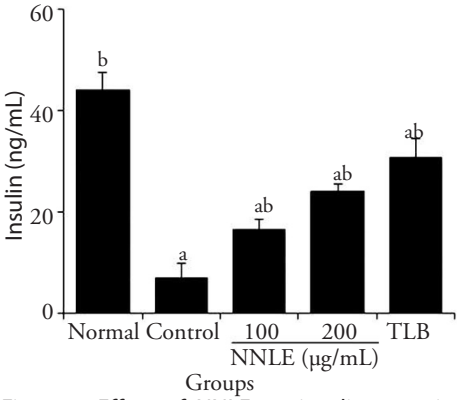

Figure 3 Effect of NNLE on insulin secretion in IL-1β and IFN-γ-induced insulinoma cells

Insulinoma cells (2×10^6) were pretreated with the indicated concentrations of NNLE for 3 h, followed by stimulation with IL-1β (2 ng/mL) and IFN-γ (100 U/mL) for 24 h. Insulin secretion was determined in cell-free culture supernatant using a rat insulin ELISA Kit. Normal: untreated cells; Control: cytokine treated cells; NNLE (µg/mL): cytokine and NNLE treated cells; TLB: tolbutamide (100 µg/mL) treated cells. NNLE: Nelumbo Nucifera leaf extract; IL-1β: interleukin-1β; IFN-γ: interferon-g; ELISA: enzyme-linked immuno sorbent assay. Each value represents the mean ± standard deviation of three independent experiments. $^aP < 0.05$, versus normal group and $^bP < 0.05$, versus control group.

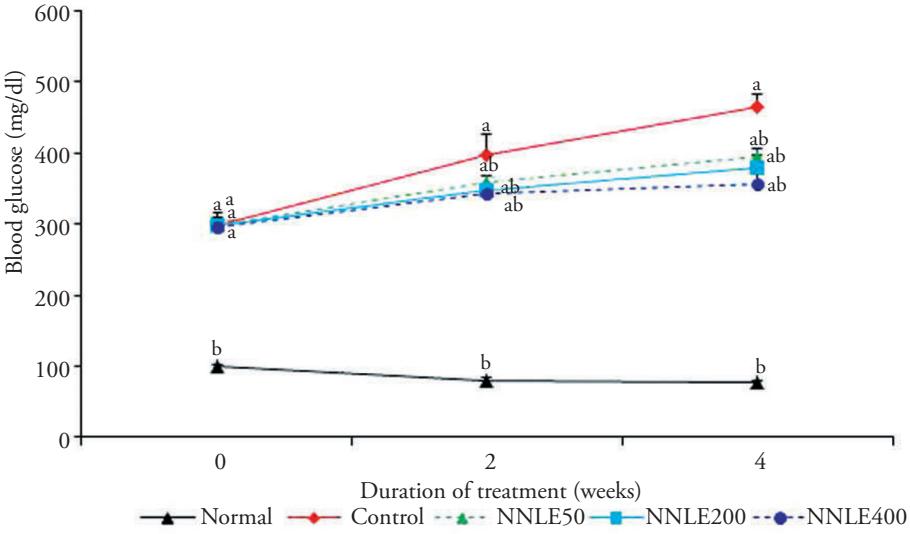

Figure 4 Effect of NNLE on blood glucose in STZ-induced diabetic rats

Normal: non-diabetic healthy rats; control: streptozotocin induced diabetic rats; NNLE50: diabetic rats treated with NNLE at 50 mg/kg body weight; NNLE200: diabetic rats treated with NNLE at 200 mg/Kg body weight; NNLE400: diabetic rats treated with NNLE at 400 mg/kg body weight. NNLE: Nelumbo Nucifera leaf extract; STZ: streptozotocin. Values are given as mean ± standard deviation for each group of six animals. $^aP < 0.05$, versus normal group and $^bP < 0.05$, versus control group.

tected RIN cells innduced by IL-1β and IFN-γ, and decreased NO production. Furthermore, it increased insulin secretion in RIN cells induced by IL-1β and IFN-γ. NNLE significantly decreased glucose and lipid levels, and prevented body weight loss in STZ-induced diabetes rats. It is suggested that, during the pathogenesis of diabetes, pancreatic β-cells generate excessive cytokines, that increase NO production and impair insulin secretion.[3] In our cell culture system, we exposed pancreatic beta cells to a mixture of cytokines, and then measured NO production, cell death, and insulin secretion. This mechanism is similar to the destruction of pancreatic β-cells in type 1 diabetic patients. Recently, several anti-diabetic plant extracts were reported to have β-cell protective effects that finally regulate glucose metabolism.[15] Sharma et al reported that Lespedeza davurica extract decreased NO production in cytokine treated RIN cells, and protected pancreatic β-cells in diabetic rats.[16] However, no one has yet reported the β-cell protective effects of NNLE. In this study, we found that NNLE protected RIN cells from injury caused by the toxicity induced IL-1β and IFN-γ. Furthermore, NNLE dose dependently decreased NO production and increased insulin secretion. Insulin secretion is partly influenced by DPP-IV. DPP-IV degrades incretin hormones, such as glucagon like peptide, and gastric inhibitory polypeptide, that play an important role in insulin secretion from pancreatic beta cells.[17] Therefore, DPP-IV inhibitors are undergoing clinical trial as insulin secreting drugs over the past few years.[18] NNLE strongly inhibited DPP-IV, suggesting that insulin secretory effect of NNLE might be partly due to the DPP-IV inhibition. Cytokine-stimulated pancreatic beta cells are vulnerable to the cytotoxic action of free radicals, because they have low level of antioxidant enzymes.[5] In our *in vitro* study, NNLE strongly decreased DPPH and NO free radical formation. We speculated that the free radical scavenging effects of NNLE could help to protect β-cells from injury induced by IL-1β and IFN-γ. It is reported that the natural remedies which protect pancreatic β-cells could inhibit α-glucosidase.[14] We also found that NNLE strongly inhibited α-glucosidase, in vitro. Our results are consistent with previous findings by Huang and his colleagues who found the insulin secretory effect of 100% MeOH extract of lotus in β-cell derived HIT-T15 cells grown in high glucose medium, and further added beta cell protective and insulin secretory ef-

Table 1 Effect of NNLE on body weight, kidney weight, urine volume and serum TG, TC, BUN, and creatinine levels in STZ-induced diabetic rats

Group	Body weight		Kidney weight (g/100g BW)	Urine volume (mL/day)	TG (mg/dL)	TC (mg/dL)	BUN (mg/dL)	Creatinine (mg/dL)
	Initial (g)	Final (g)						
Normal	272±5.892	348±6.667[b]	0.703±0.020[b]	5.400±1.065[b]	39.750±4.553[b]	62.800±4.375[b]	19.020±0.416[b]	0.490±0.010[b]
Control	228±5.963	201±6.113[a]	1.264±0.037[a]	163.333±9.615[a]	446.200±64.275[a]	129.600±7.257[a]	38.775±2.425[a]	0.570±0.031[a]
NNLE50	229±2.784	207±5.394[a]	1.274±0.024[a]	142.000±6.782[a,b]	441.750±25.181[a]	110.500±2.062[a,b]	38.680±2.046[a]	0.496±0.007[a]
NNLE200	224±3.116	216±8.110[a]	1.189±0.027[a,b]	140.000±4.163[a,b]	348.250±49.348[a]	108.250±5.648[a,b]	32.900±2.257[a,b]	0.460±0.009[a,b]
NNLE400	225±3.235	228±5.000[a,b]	1.197±0.023[a,b]	129.000±16.523[a,b]	427.250±29.596[a,b]	105.000±7.937[a,b]	32.883±1.549[a,b]	0.468±0.007[a,b]

Notes: normal: non-diabetic healthy rats; control: streptozotocin induced diabetic rats; NNLE50: diabetic rats treated with NNLE at 50 mg/kg body weight; NNLE200: diabetic rats treated with NNLE at 200 mg/Kg body weight; NNLE400: diabetic rats treated with NNLE at 400 mg/kg body weight. NNLE: Nelumbo Nucifera leaf extract; TG: triglyceride; TC: total cholesterol; BUN: blood urean nitrogen; STZ: sterptozotocin. Values are given as mean ± standard deviation for each group of six animals. [a]$P < 0.05$, versus normal group and [b]$P < 0.05$, versus control group.

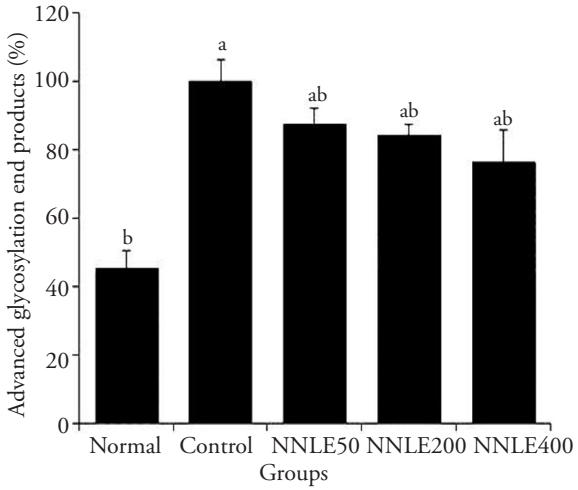

Figure 5 Effect of NNLE on AGEs in STZ-induced diabetic rats
Normal: non-diabetic healthy rats; Control: streptozotocin induced diabetic rats; NNLE50: diabetic rats treated with NNLE at 50 mg/kg body weight; NNLE200: diabetic rats treated with NNLE at 200 mg/Kg body weight; NNLE400: diabetic rats treated with NNLE at 400 mg/kg body weight. NNLE: Nelumbo Nucifera leaf extract; AGEs: advanced glycosylation end products; STZ: streptozotocin. Values are given as mean ± standard deviation for each group of six animals. [a]$P < 0.05$, versus normal group and [b]$P < 0.05$, versus control group.

fect of the water extract of Nelumbo Nucifera leaves on RIN cells induced IL-1β and IFN-γ.[9] Water extract can be used to make both functional food and herbal medicine for the prevention and therapy of diabetes. STZ-induced diabetes has been used as a useful experimental model to investigate the hypoglycemic effects of different natural remedies; it causes selective toxicity to pancreatic β-cells, which is mediated through the release of NO.[19] Selective toxicity to pancreatic β-cells by STZ causes lipid and glucose abnormalities, severe weight loss, increased food intake, and greater urine volume.[15] Loss of body weight could have resulted from protein turnover and muscle waste due to insulin deficiency. That decreased renal dysfunction, by increasing the level of serum creatinine and blood urea nitrogen, which are the energy building blocks in the muscles.[20] Hypertriglyceridemia and hypercholesterolemia are the primary factors involved in the development of atherosclerosis and coronary heart disease in diabetic patients.[20] It is recently reported that natural remedies with high flavonoid content decrease serum TG, TC, BG, BUN, uric acid, and creatinine, by protecting pancreatic β cells in STZ-induced diabetic rats.[21] Moreover, Sharma *et al* [22] recently reported that Nelumbo nucifera at 400 mg/kg body weight significantly decreased triglyceride and total cholesterol level in high fat diet induced obese mice. In agreement with these recent reports, safe dose of NNLE, which also contains high flavonoids[11], significantly decreased the levels of TG, TC, BUN, and creatinine, which may have been due to the regulation of nucleic acid and protein metabolism, owing to better glycemic control and body weight. Decreased serum AGE — which is an special marker of diabetes — in sample treated group suggested that NNLE has an effect on decreasing chronic markers of diabetes. Moreover, NNLE decreased urine volume and kidney weight, compared with STZ-induced diabetic rats. Thus, it is reasonable to conclude that NNLE protected pancreatic beta cells from toxicity induced by IL-1β and IFN-γ and regulated glucose metabolism in diabetic rats.

In summary, our study revealed, for the first time, that Nelumbo Nucifera leaf was effective in protecting rat's insulinoma cells from I death induced by IL-1β and IFN-γ. NNLE further regulated glucose and lipid metabolism in STZ-induced diabetic rats. Our results validate the traditional use of Nelumbo Nucifera leaves for the management of diabetes.

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