Natural Product Sciences
22(3) : 175-179 (2016)
http://dx.doi.org/10.20307/nps.2016.22.3.175

Beneficial Effect of *Lespedeza cuneata* (G. Don) Water Extract on Streptozotocin-induced Type 1 Diabetes and Cytokine-induced Beta-cell Damage

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Abstract – The aim of this study was to evaluate the anti-diabetic effects of the water extract of *Lespedeza cuneata* (LCW) using rat insulinoma (RIN) m5F cells and streptozotocin (STZ)-induced diabetic rats. The effect of LCW on the protection of pancreatic beta cells was assessed using MTT assay, and nitric oxide production was assessed using Griess reagent. STZ-induced diabetic rats were treated with 100 and 400 mg/kg body weight of LCW for 5 weeks. In results, LCW significantly protected cytokine-induced toxicity and NO production, and increased insulin secretion in RINm5F cells. LCW significantly decreased serum blood glucose, thiobarbituric acid reactive substances (TBARS), blood urea nitrogen (BUN) and advanced glycation end products (AGEs) levels, and renal fibronectin expression in STZ-induced diabetic rats. Also, LCW effectively improved BW loss in STZ-induced diabetic rats. Thus, our results suggest that LCW has a beneficial effect on cytokine-induced pancreatic beta cell damage and biomarkers of diabetic complication in hyperglycemic rats.
Keywords – Cytokines, Diabetes, *Lesepdeza cuneata*, Pancreatic beta cells, Streptozotocin

Introduction

Insulin dependent diabetes mellitus (IDDM) results from the selective destruction of pancreatic beta cells, and is characterized by high glucose in the blood. During the pathogenesis of IDDM, infiltration of immune cells and the generation of free radicals, in and around the islets, generate excessive cytokines such as interleukin (IL)-1β and interferon (IFN)-γ that up regulate nitric oxide (NO) production, causing the selective destruction of pancreatic beta cells.[1] Hence, rat insulinoma (RIN) m5F cells that show cellular response against the cytokine treatment in their cultured conditions have been a popular *in-vitro* model for screening the beta cell protective effects of natural remedies.[2] Streptozotocin (STZ), which is commonly used to induce diabetes, has a long history of use in diabetes research, because it is known to be specifically toxic to pancreatic beta cells as a result of alkylation and cleavage of the DNA.[3] Thus, protection of pancreatic beta cells would have a crucial role for maintaining glucose homeostasis and preventing diabetic complications. However, no medicines have been reported safe for long-term use. Therefore, interests in the use of folk medicines, which are safe or less toxic than synthetics, have recently been increased.[4] The diverse plants have long been used in South Korea for the treatment and prevention of diabetes.

Lespedeza cuneata (G. Don), a common native plant, is distributed in Korea, China, Taiwan, India, Australia, and many states of the USA. Several bioactive compounds including flavonoids, D-fructose, D-pinitol, sterols, and catechins have been isolated from *L. cuneata*.[5] It has been traditionally used for the prevention and treatment of various biological disorders including diabetes, impotence, involuntary emission of semen, and coughing and asthma for thousands of years.[6] However, the scientific evidences against diabetes are still uncertain. In our previous study, we discovered antioxidant and anti-diabetic effects of *L. cuneata* in cell free system.[7] Thus, the aim of this study is to examine the beneficial effect of *L. cuneata* on STZ-induced type 1 diabetes and cytokine-induced beta cell damage.

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Experimental

Preparations of LCW – The whole plant (herb, stem, and flower) of *L. cuneata* was collected in August of 2011 from the Geumsan (Chungnam), Korea. Professor Hui Kim confirmed the taxonomic identity of the plant, and the voucher specimen was deposited in the herbarium of the oriental medicine resources, Mokpo National University, South Korea. It was dried in an oven at 40 ºC for 24 h, and 315 g of dried form was boiled with water for 1 h. The extract was then concentrated by evaporation using rotary evaporator under reduced pressure to obtain the viscous residue, which was converted to powder form by freeze drying. The yield of water extract was about 20% of the starting material. The concentration of total phenolic acids and flavonoids in *L. cuneata* water extract (LCW) was measured as described previously.[8]

Cell culture, cell viability, NO production, and insulin secretion – Cell culture, cell viability, NO production, and insulin secretion in RINm5F cells was performed as mentioned previously.[9]

Experimental animals and induction of diabetes – Healthy male Wistar rats (120 - 250 g), purchased from the Central Lab Animal Inc. (Seoul, Korea), were maintained under standard light (12/12-h light/dark) and temperature conditions (22 ± 2 ºC). Procedures involving the animal care were conducted in conformity with the institutional guidelines of Mokpo National University, Korea. Diabetes was induced in overnight-fasted rats by a single intravenous injection of STZ (50 mg/kg body weight), freshly dissolved in cold citrate buffer (pH 4.5). Normal animals received only citrate buffer. Rats were tested for successful induction of diabetes, at 72 h after STZ induction, by measuring the fasting blood glucose level. Only those rats having blood glucose level > 250 mg/dL were used in the study.

Experimental design – The animals were assigned randomly into four groups of six animals each. LCW was dissolved in distilled water and was administered orally every day for 5 wk. Group I served as normal rats [Normal]; Group II served as STZ-induced diabetic rats [Control]; Group III served as diabetic rats orally treated with LCW (100 mg/kg body weight/day) [LCW100]; and Group-IV served as diabetic rats orally treated with LCW (400 mg/kg body weight/day) [LCW400]. Body weight was measured twice per week during the experimental period. At the end of the experiment, overnight fasted rats were anesthetized with diethyl ether and blood was collected from the abdominal artery for various biochemical analyses.

Measurement of blood glucose – The blood glucose level was measured each week after overnight fasting using blood glucose test meter (GlucoDr, Almedicus, Korea).

Biochemical analysis – Collected blood was immediately centrifuged at 3000 rpm for 20 minutes to separate the serum from the blood cells; the levels of serum total protein, blood urea nitrogen (BUN), and advanced glycation end products (AGEs) were measured spectrophotometrically, using commercially available kits. Malondialdehyde (MDA), an end product of unsaturated fatty acid peroxidation, which can react with thiobarbituric acid (TBA) to form colored complex thiobarbituric acid reactive substances (TBARS), was measured using spectrophotometer. The expression of renal fibronectin protein was performed as mentioned previously.[9]

Statistical analysis – The statistical significance was evaluated by one-way analysis of variance (ANOVA) using the SPSS/17.0 software followed by the Duncan's post hoc tests. Data represent the mean ± SE for all the experiments. P values < 0.05 were considered statistically significant.

Result and Discussion

Components of LCW – Major bioactive compounds, including flavonoids, D-fructose, D-pinitol, sterols, and catechins, have been isolated from *L. cuneata*, which are reported to have anti-diabetic activities.[5] In this study, LCW contained high polyphenols (65 mg equivalent of gallic acid/g of the extract) and flavonoids (80 mg equivalent of rutin/g of the extract). It has been reported that gallic acid and rutin decreased blood glucose and increased insulin secretion in streptozotocin-induced diabetic rats by altering glycolytic and gluconeogenetic enzymes.[10,11] Therefore, we speculated that gallic acid and rutin with other anti-diabetic compounds present in LCW might have decreased blood glucose level in STZ induced diabetic rats.

Effects of LCW on IL-1β and IFN-γ induced cell death, NO production, and insulin secretion – RINm5F cells were treated with various concentrations of LCW to assess its cytotoxicity (data not shown). Then, non-toxic doses of LCW (50 and 100 μg/mL) were used for subsequent experiments. Fig. 1 depicts that the combination of IL-1β and IFN-γ decreased cell viability (52.8%) and insulin secretion (8.7 ng/mL), and increased NO production (70.4 μM) compared to normal (100%, 44.6 ng/mL, and 9.5 μM). During the pathogenesis of diabetes, pancreatic beta cells generate excessive cytokines and free radicals that increase NO production causing beta cell death.[1] In our cell cultured system, the concentration of 50 and 100 μg/mL of LCW significantly increased cell viability to 67.0 and 73.0%, and decreased NO production to 58.6 and

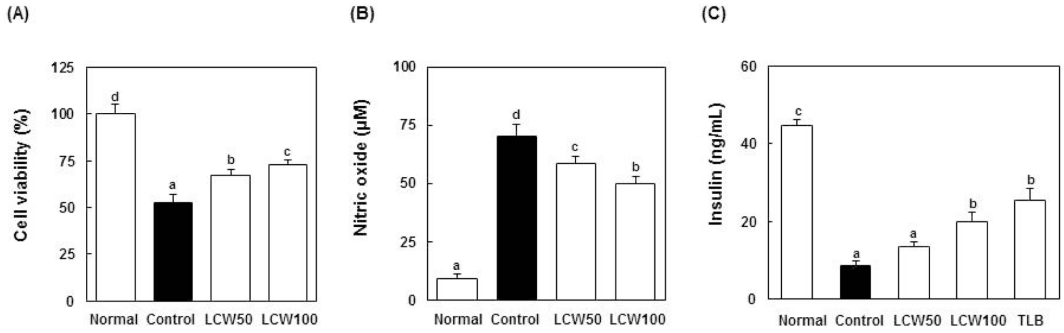

Fig. 1. Effects of LCW on IL-1β- and IFN-γ-induced cell viability (A), NO production (B), and insulin secretion (C) in RINm5F cells. RINm5F cells (2×10^6) were pretreated with the indicated concentrations (50 and 100 μg/mL) of LCW for 3 h, followed by stimulation with IL-1β (2 ng/mL) and IFN-γ (100 U/mL) for 48 h. 100 μM TLB was used as a positive control. Each value represents the mean ± SE of three independent experiments. Bars with different letters (a, b, c, and d) are significantly different each other at $p < 0.05$ in Duncan's multiple comparison tests.

50.2 μM, respectively (Fig. 1). Insulin secretion significantly increased to 13.4 and 19.9 ng/mL (Fig. 1). Many studies suggested that plant extracts, which have NO inhibitory activities, may regulate blood glucose in STZ-induced diabetic rats.[12] Consequently, we further discovered whether LCW might regulate glucose homeostasis in STZ-induced diabetic rats.

Effects of LCW on blood glucose and body weight in diabetic rats – In our previous study, we found the free radical scavenging, and dipeptidyl peptidase (DPP)-IV and alpha glucosidase enzyme inhibitory activity of LCW.[7] Recently, several studies have suggested that natural remedies that inhibit α-glucosidase and DPP-IV could regulate glucose metabolism and insulin secretion in diabetic conditions.[9] Therefore, we hypothesized that LCW might decrease blood glucose in STZ-induced diabetic model. STZ-induced diabetes has been used as a useful experimental model to investigate the hypoglycemic effect of different natural remedies; it causes selective toxicity to pancreatic beta cells, causing hyperglycemia and severe weight loss.[9,13] STZ-induced diabetic rats showed a significant increase in the levels of blood glucose compared to normal rats (Fig. 2). After treatment with LCW, the blood glucose was significantly reduced from third week of the experiment (Fig. 2). Body weight is significantly decreased in diabetic rats (Table 1). However, the oral administration of LCW (100 and 400 mg/kg body weight) showed weight gain compared to STZ-induced diabetic rats (Table 1). Loss of body weight could result from protein turnover and muscle waste due to insulin deficiency. We have previously reported that *L. davurica* prevents body weight loss by protecting pancreatic beta cells in STZ-induced diabetic rats.[9] However, further studies are needed to understand the detailed mechanisms of how

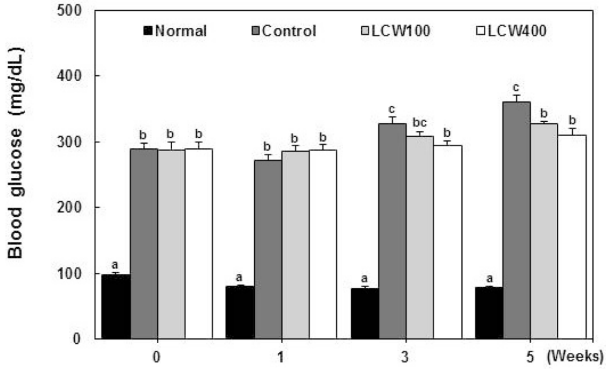

Fig. 2. Effect of LCW on blood glucose in STZ induced diabetic rats. Values are given as mean ± SE for each group of six animals. Normal, normal rats; control, diabetic control rats, LCW100, treatment with LC extract at dose of 100 mg/kg BW; LCW400, treatment with LC extract at dose of 400 mg/kg BW. Bars with different letters (a, b, and c) are significantly different each other at $p < 0.05$ in Duncan's multiple comparison tests.

LCW regulates blood glucose in diabetic rats.

Effects of LCW on biochemical features of diabetic complications in diabetic rats – As shown in Table 1 and Fig. 3, serum BUN, TBARS, and AGEs levels and expression of renal fibronectin are significantly increased and serum total protein is significantly decreased in STZ-induced diabetic rats compared to normal rats. However, oral administration of LCW (100 and 400 mg/kg body weight) effectively decreased serum BUN, TBARS and AGEs levels and renal fibronectin expression, and increased the level of total protein with dose-dependent manners (Table 1 and Fig. 3). TBARS are considered as a marker of endogenous lipid peroxidation and are increased in diabetic patients.[14] However, our body has a complex antioxidant defense system that prevents the initiation of free radical chain reactions. Herein, LCW decreased the level TBARS in the serum of LCW administered diabetic

Table 1. Effects of LCW on body weight, serum total protein, BUN, TBARS, and AGEs levels in STZ-induced diabetic rats

Group	Body weight (g)			Serum Total Protein (g/dL)	Serum BUN (mg/dL)	Serum TBARS (nM/mg protein)	Serum AGEs (nM/g protein)
	Initial	Final	Gain				
Normal	280 ± 4	377 ± 9	97 ± 5^{b}	5.6 ± 0.1^{c}	19.3 ± 0.8^{a}	26.9 ± 3.9^{a}	180 ± 12.1^{a}
Control	263 ± 10	245 ± 17	-18 ± 9^{a}	4.9 ± 0.1^{ab}	33.9 ± 3.6^{b}	159.0 ± 24.7^{b}	263 ± 22.9^{b}
LCW100	261 ± 6	257 ± 16	-4.5 ± 11^{a}	4.7 ± 0.2^{a}	30.4 ± 2.4^{b}	133.3 ± 27.1^{b}	240 ± 15.0^{b}
LCW400	265 ± 7	261 ± 11	-4 ± 5^{a}	5.1 ± 0.1^{b}	29.9 ± 3.6^{b}	118.4 ± 32.1^{b}	204 ± 50.3^{b}

Normal, normal rats; control, diabetic control rats, LCW100, treatment with LC extract at dose of 100 mg/kg BW; LCW400, treatment with LC extract at dose of 400 mg/kg BW. Values are means ± SE for each group of six animals. Values with different letters (a and b) are significantly different each other at $p < 0.05$ in Duncan's multiple comparison tests.

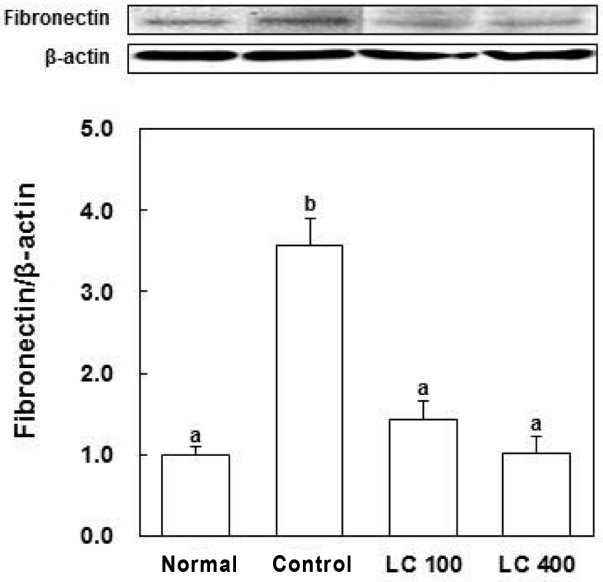

Fig. 3. Effect of LCW on renal fibronectin expression in STZ-induced diabetic rats. Normal, normal rats; control, diabetic control rats, LCW100, treatment with LC extract at dose of 100 mg/kg BW; LCW400, treatment with LC extract at dose of 400 mg/kg BW. Bars with different letters (a and b) are significantly different each other at $p < 0.05$ in Duncan's multi comparison tests.

rats revealing its lipid peroxidation inhibitory activity. Chronic hyperglycemia in diabetes assists the reaction between glucose and amino group of proteins, lipids, and nucleic acids forming AGEs, which induces injury to pancreatic beta cells.[15] It further decreases renal dysfunction and increases the level of serum creatinine and blood urea nitrogen. It has been reported that a high BUN correlates with decreased kidney function in diabetes, but other factors may also affect the BUN level.[16] Therefore, diabetic nephropathy may start when the level of BUN rises. Herein, LCW decreased the BUN in STZ-induced diabetic rats revealing its effect in diabetic complications. The accumulation of extracellular matrix (ECM) proteins such as fibronectin is the main feature of diabetic complications.[17] Therefore, LCW may subside the development of diabetic complications through regulation of blood glucose in STZ-induced diabetic rats.

In conclusion, this is the first findings to show the anti-diabetic effects of LCW using cell and animal models of diabetes. LCW protected the destruction of cytokine-induced pancreatic beta-cells, and increased insulin secretion *via* inhibiting NO production. Also, LCW controlled blood glucose, and protected renal dysfunction through regulation of diverse biochemical and physiological factors in STZ-induced diabetic rats. Our results support the traditional use of *L. cuneata* as an anti-diabetic folk medicine.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (NRF-2009-0077541).

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Received January 6, 2016
Revised February 29, 2016
Accepted February 29, 2016