

Protective effects of *Lagerstroemia speciosa* on 3-morpholinosydnonimine (SIN-1)-induced oxidative stress in HIT-T15 pancreatic β cells

JIA-LE SONG^{1*}, XIN ZHAO^{1,2}, QIANG WANG² and TING ZHANG^{3*}

¹Department of Food Science and Nutrition, Pusan National University, Busan 609-735, Republic of Korea;

²Department of Biological and Chemical Engineering, Chongqing University of Education, Chongqing 400067;

³Research Center for Nutrition and Food Safety, The Third Military Medical University, Chongqing Key Laboratory of Nutrition and Food Safety, Chongqing 400038, P.R. China

Received November 15, 2012; Accepted March 19, 2013

DOI: 10.3892/mmr.2013.1396

Abstract. Reactive oxygen species (ROS)-induced pancreatic β cell death affects insulin secretion and is important in the pathogenesis of diabetes. *Lagerstroemia speciosa*, a traditional folk medicine, has been used for the prevention and treatment of diabetes. However, whether *Lagerstroemia speciosa* has a cytoprotective effect on pancreatic β cells remains to be elucidated. The present study aimed to investigate the cytoprotective effects of hot water extracts from *Lagerstroemia speciosa* leaves (LWE) on 3-morpholinosydnonimine (SIN-1)-induced oxidative damage in Syrian hamster pancreatic insulinoma HIT-T15 cells. The HIT-T15 cells were first treated with SIN-1 (50 μ M) for 24 h and then co-incubated with LWE for 48 h. SIN-1 significantly decreased HIT-T15 cell viability ($P < 0.05$); however, LWE did not exert a significant cytotoxic effect and increased the viability of HIT-T15 cells in a dose-dependent manner. To further investigate the protective effects of LWE on SIN-1-induced oxidative stress in HIT-T15 cells, the cellular levels of ROS, lipid peroxidation and endogenous antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-px), were determined. LWE decreased the intracellular levels of ROS and lipid peroxidation, and increased the activities of antioxidant enzymes. These results suggest that LWE has a cytoprotective effect against SIN-1-induced oxidative stress in HIT-T15 cells through the inhibition of lipid peroxidation, a decrease in ROS levels and an increase in antioxidant enzyme activity. In addition, LWE increased insulin secretion in SIN-1-treated

HIT-T15 cells. Our results suggested that LWE were effective in the treatment of diabetes. Further studies are required to study the anti-diabetic molecular mechanism in a cell model.

Introduction

Diabetes mellitus has become an increasingly important public health problem worldwide. However, the etiology of diabetes mellitus has not yet been fully elucidated. Reactive oxygen species (ROS)-induced oxidative stress is known to be important in the pathogenic process of diabetes mellitus. ROS that are particularly responsible for oxidative stress include superoxide (O_2^-), hydroxyl radical ($\cdot OH$), singlet oxygen (1O_2), hydrogen peroxide (H_2O_2), nitric oxide (NO) and peroxynitrite ($ONOO^-$) (1). Oxidative stress may induce the dysfunction of pancreatic β cells, decreased insulin secretion (2) and the development of diabetic complications, including retinopathy, nephropathy, neuropathy and vascular damage (3,4). Generally, various antioxidative compounds exist in mammalian cells, including low-molecular mass antioxidants such as glutathione (GSH), uric acid, vitamin C, vitamin E and various endogenous antioxidant enzymes against oxidative stress. It is widely accepted that superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-px) are three important endogenous antioxidant enzymes for the protection of living organs against ROS-induced oxidative stress. Among these antioxidant enzymes, SOD catalyzes the dismutation of O_2^- into H_2O_2 , which may be transformed into H_2O and O_2 by CAT. GSH-px is crucial for removing lipid hydroperoxides and reducing free H_2O_2 to water (1).

A number of drugs used in clinical diabetes mellitus treatment have been associated with side-effects, including gastrointestinal disturbances, edema, myocardial infarction and risk of cardiovascular disease (5-8). To date, >400 traditional plant treatments for diabetes mellitus have been identified (9). The anti-diabetic components of these natural plants may constitute ancillary medication for diabetes treatment.

Lagerstroemia speciosa (Lythraceae), also named banaba, is a tropical plant that grows in several parts of southeast Asia, including southern China, Vietnam,

Correspondence to: Dr Xin Zhao, Department of Biological and Chemical Engineering, Chongqing University of Education, No. 9 Xuefu Main Street, Nan'an, Chongqing 400067, P.R. China
E-mail: zhaoxin@pnu.edu

*Contributed equally

Key words: *Lagerstroemia speciosa*, pancreatic β cell, cell viability, reactive oxygen species, insulin secretion

Malaysia and the Philippines. *Lagerstroemia speciosa* has been used as a traditional folk medicine for the treatment of diabetes and kidney-related diseases in the Philippines for ~1,000 years (10,11). A number of studies have reported that *Lagerstroemia speciosa* has antioxidant (12,13), anti-inflammatory (13), anticancer (14), anti-obesity (15) and antidiabetic (12,16) activities. Tannic and triterpene acids are the main components of *Lagerstroemia speciosa* leaf extracts and have been shown to downregulate blood glucose and possess apparent antidiabetic properties *in vivo* and *in vitro* (16-19).

The present study aimed to investigate the potential cytoprotective effects of hot water extracts from *Lagerstroemia speciosa* leaves (LWE) on 3-morpholinosydnnonimine (SIN-1)-induced oxidative stress in HIT-T15 cells and to elucidate the underlying mechanisms involved in this process.

Materials and methods

Plant extract preparation. Fresh *Lagerstroemia speciosa* leaves were purchased from a local market in Chongqing, China. LWE was prepared by boiling 160 g air-dried *Lagerstroemia speciosa* leaves in 1 l distilled water for 2 h, followed by ultracentrifugation at 30,000 x g for 30 min, filtration with a 0.4- μ m filter, concentration by heat evaporation and freeze-drying. LWE was redissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/ml and stored at 4°C until future use.

Cell culture. Syrian hamster insulin-secreting HIT-T15 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were routinely maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified CO₂ incubator (model 3154; Forma Scientific, Inc., Marietta, OH, USA) with 5% CO₂ at 37°C.

Cell viability assay. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. The cells were seeded in 96-well plates at a density of 5x10³ cells/well. Following 24-h incubation, the cells were primarily treated with SIN-1 (50 μ M) for 24 h and then incubated with LWE (1-100 μ g/ml) for 48 h. Next, 100 μ l MTT reagent (final concentration, 0.5 mg/ml) was added to each well and the cells were incubated in a humidified incubator at 37°C to allow MTT to be metabolized. After 4 h, 100 μ l DMSO was added to each well to dissolve formazan deposits. The absorbance of the samples was measured at a wavelength of 540 nm using a microplate reader (model 680; Bio-Rad, Hercules, CA, USA).

Determination of intracellular ROS. Intracellular ROS levels were measured using the fluorescent probe dihydrodichlorofluorescein (H₂DCFDA). Following treatment, HIT-T15 cells were washed with calcium- and magnesium-free phosphate-buffered saline (PBS) and incubated in H₂DCFDA (20 μ M) containing serum- and phenol red-free Dulbecco's modified Eagle's medium (DMEM) for 30 min. Following incubation, the medium was removed and cells were washed with PBS twice. Fluorescence was measured using a FLUOstar

OPTIMA fluorescence plate reader (BMG Labtech, Ortenberg, Germany); excitation was read at 485 nm and emission at 535 nm. Relative ROS production (calculated as a percentage of the control) was expressed as the ratio of fluorescence in the treated samples over the response in the appropriate controls: (fluorescence_{treatment}/fluorescence_{control}) x100.

Lipid peroxidation levels. Lipid peroxidation was evaluated using a thiobarbituric acid reactive substance (TBARS) assay (20). Briefly, the cultured cells were washed with cooled PBS, scraped into trichloroacetic acid (TCA; 2.8%, w/v) and sonicated; total protein was determined using a bicinchoninic acid (BCA) assay. The suspension was mixed with 1 ml TBA (0.67%, w/v) and 1 ml TCA (25%, w/v), heated (30 min at 95°C) and centrifuged (22,000 x g; 10 min at 4°C). TBA reacted with the oxidative degradation products of lipids, yielding red complexes that absorbed at 535 nm. The level of TBARS was determined using a UV-2401PC spectrophotometer (Shimadzu, Kyoto, Japan).

Antioxidant enzyme activity. HIT-T15 cells grown in a 10-cm cell culture dish were co-incubated with SIN-1 (50 μ M) for 24 h and then treated with LWE (2.5-50 μ g/ml) for 48 h for further assessment. The cells were washed with PBS, removed by scraping and centrifuged, and the resulting cell pellet was stored at -80°C. Cell pellets were thawed, resuspended in 300 μ l cold lysis buffer (PBS and 1 mM EDTA), homogenized and centrifuged (22,000 x g; 10 min at 4°C). The resulting supernatants were used for activity measurements. CAT activity (U/mg protein) was assessed according to the method described by Nelson and Kiesow (21), in which the disappearance of the substrate H₂O₂ was measured spectrophotometrically at 240 nm. SOD activity (U/mg protein) was assayed using a modified auto-oxidation of pyrogallol method (22). One unit of SOD activity was defined as the amount of enzyme that inhibited the auto-oxidation rate of pyrogallol by 50%. GSH-px activity (U/mg protein) was assayed according to the method described by Hafemen *et al.* (23). Protein contents were determined using the Bio-Rad protein assay kit according to the manufacturer's instructions.

Insulin secretion assay. Insulin secretion was measured using a radioimmunoassay (RIA) method. The cells were seeded at a density of 5x10⁵ cells/well in a 96-well plate and primarily treated with SIN-1 (50 μ M) for 24 h, followed by LWE (2.5-50 μ g/ml) for 48 h. To determine the level of insulin secreted, aliquots of samples (10 μ l/well) were collected from the experimental medium at the indicated time points (48 h) and subjected to an insulin antiserum immunoassay within 5 min, according to the manufacturer's instructions (Linco Research, Inc., St. Charles, MO, USA). The absorbance was read at 450 and 590 nm using a model 680 microplate reader (Bio-Rad) and results were recorded.

Statistical analysis. Data are presented as the mean \pm SD. The differences between the mean values of individual groups were assessed using one-way ANOVA and Duncan's multiple range tests. P<0.05 was considered to indicate a statistically significant difference. The SAS v9.1 statistical software package (SAS Institute, Inc., Cary, NC, USA) was used for analysis.

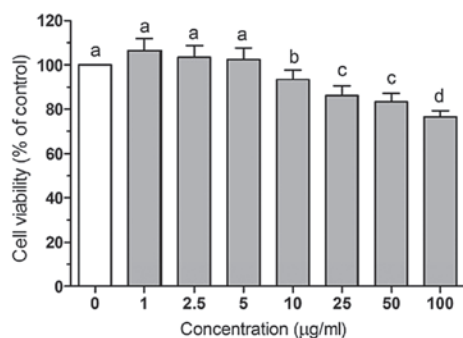


Figure 1. Effects of hot water extracts from *Lagerstroemia speciosa* leaves (LWE) on the cell viability of HIT-T15 pancreatic cells. Data are representative of three independent experiments and are expressed as the mean \pm SD. ^{a-d}Mean values with different letters on the bars are significantly different ($P<0.05$) according to Duncan's multiple range test.

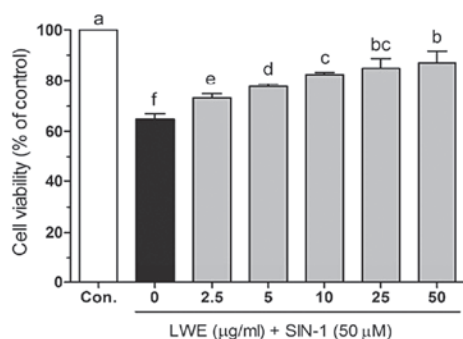


Figure 2. Effects of hot water extracts from *Lagerstroemia speciosa* leaves (LWE) on the cell viability of SIN-1-treated HIT-T15 pancreatic cells. Data are representative of three independent experiments and are expressed as the mean \pm SD. ^{a-f}Mean values with different letters on the bars are significantly different ($P<0.05$) according to Duncan's multiple range test. SIN-1, 3-morpholinosydnnonimine.

Results

Effects of LWE on SIN-1-induced oxidative damage in HIT-T15 cells. To investigate LWE-induced cytotoxicity, HIT-T15 cells were first treated with various concentrations of LWE (1-100 µg/ml) for 48 h and the cell viability was determined using an MTT assay. Treatment with LWE at doses of 1-50 µg/ml at 37°C for 48 h did not cause significant cytotoxicity and cell viability was $>80\%$. A dose of 100 µg/ml LWE induced cell damage (cell viability, 76%; Fig. 1). Based on these results, concentrations of 2.5-50 µg/ml LWE were used for further assessment. As shown in Fig. 2, SIN-1 (50 µM) significantly induced cell death in HIT-T15 cells. However, following treatment with various concentrations of LWE, the cell viability increased in a dose-dependent manner.

Effects of LWE against SIN-1-induced intracellular ROS levels in HIT-T15 cells. To investigate the protective effects of LWE in SIN-1-treated HIT-T15 cells, the intracellular ROS levels were determined using the fluorescent probe H_2DCFDA . As shown in Fig. 3, SIN-1 significantly increased ROS levels compared with those in the control cells. In the presence of SIN-1, LWE at doses of 2.5-50 µg/ml significantly reduced

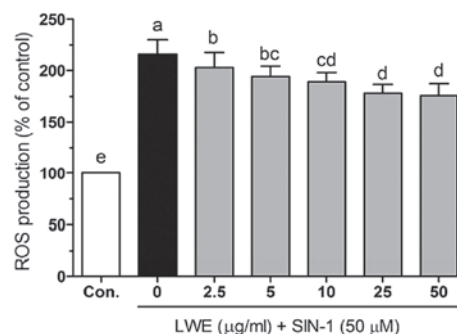


Figure 3. Effects of hot water extracts from *Lagerstroemia speciosa* leaves (LWE) on intracellular ROS levels in SIN-1-treated HIT-T15 pancreatic cells. Data are representative of three independent experiments and are expressed as the mean \pm SD. ^{a-e}Mean values with different letters on the bars are significantly different ($P<0.05$) according to Duncan's multiple range test. ROS, reactive oxygen species; SIN-1, 3-morpholinosydnnonimine.

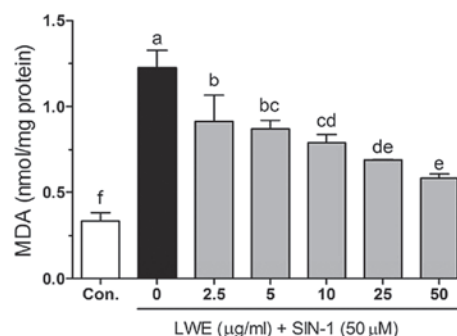


Figure 4. Effects of hot water extracts from *Lagerstroemia speciosa* leaves (LWE) on intracellular malondialdehyde (MDA) levels in SIN-1-treated HIT-T15 pancreatic cells. Data are representative of three independent experiments and are expressed as the mean \pm SD. ^{a-f}Mean values with different letters on the bars are significantly different ($P<0.05$) according to Duncan's multiple range test. SIN-1, 3-morpholinosydnnonimine.

ROS generation in a dose-dependent manner. The intracellular ROS levels were 203.2 ± 14.6 , 194.3 ± 9.9 , 188.3 ± 9.8 , 178.3 ± 7.5 and $175.7\pm10.8\%$ when the cells were treated with 2.5, 5, 10, 25 and 50 µg/ml LWE, respectively. Treatment with the same concentrations of LWE alone did not significantly increase the intracellular ROS levels (data not shown). These results suggest that LWE is a free radical scavenger.

Effects of LWE on lipid peroxidation in SIN-1-treated HIT-T15 cells. Free radical- and ROS-induced oxidative damage has been strongly associated with the lipid peroxidation of cell membranes and increased levels of malondialdehyde (MDA), which is a biomarker of cell membrane lipid peroxidation. As shown in Fig. 4, SIN-1 significantly increased the level of MDA (1.23 ± 0.10 nmol/mg protein) compared with that in the control cells (0.33 ± 0.05 nmol/mg protein). LWE at doses of 2.5-50 µg/ml significantly reduced MDA levels in a dose-dependent manner. The MDA levels were 0.91 ± 0.15 , 0.87 ± 0.05 , 0.79 ± 0.05 , 0.69 ± 0.01 and 0.58 ± 0.02 nmol/mg protein when the cells were treated with 2.5, 5, 10, 25 and 50 µg/ml LWE, respectively.

Effects of LWE on the activity of antioxidant enzymes in SIN-1-treated HIT-T15 cells. Figs. 5-7 demonstrate the intracel-

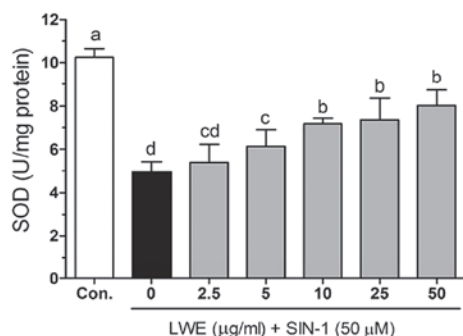


Figure 5. Effects of hot water extracts from *Lagerstroemia speciosa* leaves (LWE) on superoxide dismutase (SOD) levels in SIN-1-treated HIT-T15 pancreatic cells. Data are representative of three independent experiments and are expressed as the mean \pm SD. ^{a-d}Mean values with different letters on the bars are significantly different ($P < 0.05$) according to Duncan's multiple range test. SIN-1, 3-morpholiniosydnonimine.

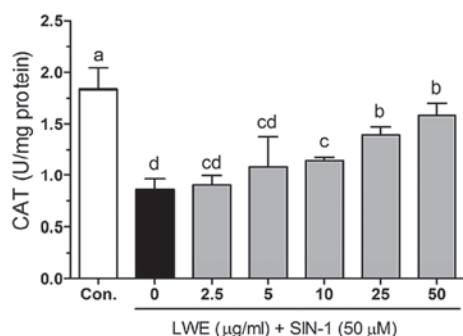


Figure 6. Effects of hot water extracts from *Lagerstroemia speciosa* leaves (LWE) on catalase (CAT) levels in SIN-1-treated HIT-T15 pancreatic cells. Data are representative of three independent experiments and are expressed as the mean \pm SD. ^{a-d}Mean values with different letters on the bars are significantly different ($P < 0.05$) according to Duncan's multiple range test. SIN-1, 3-morpholiniosydnonimine.

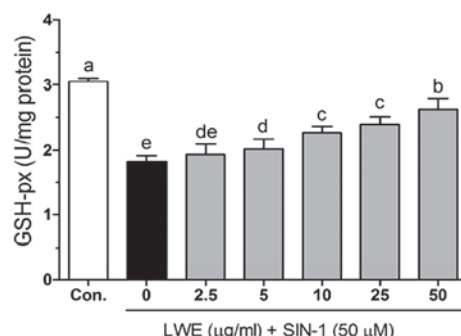


Figure 7. Effects of hot water extracts from *Lagerstroemia speciosa* leaves (LWE) on glutathione peroxidase (GSH-px) levels in SIN-1-treated HIT-T15 pancreatic cells. Data are representative of three independent experiments and are expressed as the mean \pm SD. ^{a-e}Mean values with different letters on the bars are significantly different ($P < 0.05$) according to Duncan's multiple range test. SIN-1, 3-morpholiniosydnonimine.

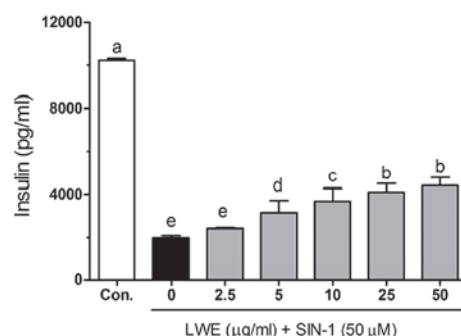


Figure 8. Effects of hot water extracts from *Lagerstroemia speciosa* leaves (LWE) on insulin levels in SIN-1-treated HIT-T15 pancreatic cells. Data are representative of three independent experiments and are expressed as the mean \pm SD. ^{a-e}Mean values with different letters on the bars are significantly different ($P < 0.05$) according to Duncan's multiple range test. SIN-1, 3-morpholiniosydnonimine.

lular antioxidant enzyme activities with LWE in SIN-1-treated HIT-T15 cells. The activity of SOD was decreased with SIN-1 treatment (4.96 ± 0.46 U/mg protein) compared with that in the control cells; however, this recovered to 5.39 ± 0.83 , 6.13 ± 0.78 , 7.19 ± 0.26 , 7.37 ± 0.99 and 8.04 ± 0.73 U/mg protein when the cells were treated with 2.5, 5, 10, 25 and 50 μ g/ml LWE, respectively. Following treatment with SIN-1, cellular CAT was decreased (0.86 ± 0.10 U/mg protein) compared with that in the control cells (1.83 ± 0.21 U/mg protein). However, CAT activity was significantly increased ($P < 0.05$) following treatment with LWE (Fig. 6). Additionally, LWE reduced the SIN-1-induced decrease in GSH-px in HIT-T15 cells. The GSH-px activity was identified to be significantly increased from 1.93 ± 0.17 to 2.63 ± 0.17 U/mg protein when the cells were treated with LWE (Fig. 7).

Effects of LWE on insulin secretion in SIN-1-treated HIT-T15 cells. As shown in Fig. 8, SIN-1 significantly decreased insulin levels (2095.4 ± 105.0 pg/ml) compared with those in the control cells (10236.7 ± 98.9 pg/ml). Following treatment with 2.5, 5, 10, 25 and 50 μ g/ml LWE, the insulin levels were 2433.7 ± 34.5 , 2824.2 ± 150.3 , 3565.4 ± 223.3 , 4730.9 ± 140.3 and 5069.2 ± 131.5 pg/ml, respectively. These results suggest that

LWE treatment is effective in increasing pancreatic β cell survival and maintaining normal biological functions in ROS-induced diabetes.

Discussion

ROS-induced oxidative damage in pancreatic β cells is considered to be important in the pathological process of diabetes. A number of studies have shown that reducing ROS levels and treatment with antioxidants (including NAC, vitamin C and vitamin E) improved β cell structure and function *in vitro* (24,25). However, whether LWE protects pancreatic β cells against SIN-1-induced oxidative damage has not yet been elucidated. In the present study, we demonstrated that LWE protected HIT-T15 cells against ROS-induced cell damage. The cytoprotective effects of LWE were mainly mediated by increased intracellular antioxidant enzyme activity.

The results of this study clearly showed that LWE prevented SIN-1-induced cell death, as assessed using the MTT assay. Additionally, LWE alone was not significantly cytotoxic to cells at the concentrations used. Treatment with LWE was shown to have a significant protective effect, which may be attributed to the free radical scavenging activity of the extract.

To evaluate the role of free radicals in the protective activity of LWE, the effect of LWE on SIN-1-induced ROS generation was analyzed using the H₂DCFDA assay. SIN-1 treatment alone significantly increased intracellular ROS generation. Following treatment with LWE, ROS generation was found to decline in a dose-dependent manner. This decrease in the SIN-1-induced ROS generation may account for the decline in the observed cytoprotective effect of LWE.

Lipid peroxidation is the most extensively investigated process induced by free radicals. ROS participate in the toxic actions that lead to apoptosis in insulin-producing cells (26). In the present study, increased lipid peroxidation levels were observed in SIN-1-treated HIT-T15 cells. However, treatment with LWE resulted in a decrease in lipid peroxidation, indicating that oxidative stress-related damage was reduced in LWE-treated cells. The ability of LWE to reduce lipid peroxidation may be due to its function as a preventive antioxidant to scavenge initiating radicals.

The overproduction and consequently increased levels of free radicals may be scavenged by endogenous antioxidant enzymes, including SOD and GSH-px. In cells, SOD catalyzes the conversion of O₂⁻ to H₂O₂, and H₂O₂ is further reduced to H₂O by the activity of CAT or GSH-px. Pancreatic β cells have been reported to contain low levels of endogenous antioxidant enzymes, particularly GSH-px and CAT (27). In the present study, SIN-1-treated HIT-T15 cells were shown to have decreased GSH-px and CAT activities, which may be due to the increased oxidative damage induced by SIN-1. However, LWE treatment caused an increase in the activity of these antioxidant enzymes in HIT-T15 cells, indicating that LWE reduced SIN-1-induced oxidative stress. A number of studies have reported that the overexpression of CuZnSOD had a protective effect in NO-induced human islets, INS-1 insulin-secreting cells (28) and alloxan- and streptozotocin-induced diabetes (29,30). CAT has also exhibited a protective effect against H₂O₂ and streptozotocin-induced oxidative stress *in vivo* (31). Additionally, combinatorial overexpression of CAT and GSH-px has been shown to have a protective effect against ROS-induced oxidative stress through improving the activity of CuZnSOD or MnSOD (32-35).

In conclusion, the present study showed that LWE had protective activity against SIN-1-induced cell death in Syrian hamster HIT-T15 insulin-secreting cells. LWE effectively scavenged the products of SIN-1-induced intracellular ROS generation and reduced pancreatic β cell death through increasing the activity of intracellular antioxidant enzymes, including SOD, CAT and GSH-Px. LWE also promoted insulin secretion in SIN-1-treated HIT-T15 cells.

Acknowledgements

This study was supported by the Natural Science Foundation Project of CQ CSTC (No. CSTC2012jjA80002) and the Science and Technology Research Project of Chongqing Municipal Education Commission (No. KJ121504)

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