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Antidiabetic Effects of Rice Hull Smoke Extract in Alloxan-Induced Diabetic Mice

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ABSTRACT: This study investigated the protective effect of a liquid rice hull smoke extract (RHSE) against diabetes in alloxan-induced diabetic mice. Antidiabetic effects of RHSE were evaluated in both the rat insulinoma-1 cell line (INS-1) and diabetic ICR mice induced by intraperitoneal (ip) injection of alloxan. Alloxan treatment (10 mM) increased cellular reactive oxygen species (ROS) levels in the INS-1 cells, which were inversely related to cell viabilities. RHSE inhibited alloxan-induced nitric oxide (NO) generation through inhibition of inducible nitric oxide synthase (iNOS) gene expression and suppressed the inflammatory reaction in INS-1 cells through inhibition of expression of pro-inflammatory genes, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). Dietary administration of 0.5 or 1% RHSE to alloxan-induced diabetic mice caused a decrease in blood glucose and increases in both serum insulin and hepatic glycogen levels. RHSE induced decreases in glucose-6-phosphatase (G6 Pase) and phosphoenolpyruvate carboxykinase (PEPCK) levels and an increase in the glucokinase (GCK) level. These changes resulted in restoring glucose-regulating enzyme levels to control values. Histopathology showed that alloxan also induced damage of Langerhans islet cells of the pancreas and liver necrosis associated with diabetes. Oral administration of RHSE restored the islet and liver cells to normal levels. RHSE-supplemented functional food could protect insulin-producing islet cells against damage triggered by oxidative stress and local inflammation associated with diabetes.

KEYWORDS: *rice hull liquid smoke, antioxidative effect, mice, oral feeding, antidiabetic effect, biomarkers, mechanism*

INTRODUCTION

Diabetes is a complex human disease characterized by overproduction of glucose by the liver and its underutilization by other organs.[1,2] The disease is caused by underproduction of insulin by the Langerhans islet cells of the pancreas and impaired insulin resistance, leading to hyperglycemia and resulting in impaired entry of glucose into cells, thus hindering glucose utilization. The aldehyde group of excess blood glucose can react in vivo with amino groups of functional and structural proteins including hemoglobin and collagen, forming Maillard glycation products that contribute to the pathogenesis of diabetes and other diseases.[3,4] Hemoglobin adduct levels can serve as an indicator of the severity of the disease. These reactions are analogous to heat-induced nonenzymatic browning in food.[5,6] It is not known whether related hemoglobin−acrylamide adducts[7] contribute to the adverse consequences of diabetes. Untreated diabetes is characterized by abnormal glucose metabolism, presumably because insulin levels are too low and glucagon levels are too high. Normal human glucose blood levels range from 80 to 120 mg/100 mL.

The pathogenesis of autoimmune β-cell destruction that contributes to the causes of diabetes is not well understood. Treatment includes control of hyperglycemia with insulin and synthetic drugs. In a previous study,[8] we described the production and composition of a new rice hull liquid smoke with a smoky aroma and sugar-like odor prepared by pyrolysis of rice hulls followed by liquefaction of the resulting smoke. The liquid smoke contained 161 compounds, as characterized by gas chromatography−mass spectrometry (GC-MS). In vitro and in vivo cell and mouse assays showed that the extract exhibited strong antioxidative, antiallergic, and anti-inflammatory activities, similar to those we previously reported for black rice bran[9,10] and medicinal mushrooms.[11,12] Related studies by other investigators showed that a rice hull extract inhibited the growth of the toxic cyanobacterium *Microcystis aeruginosa* and that far-infrared radiated rice hull extracts possessed significant reactive oxygen scavenging and protective effects against oxidative DNA damage.[13,14]

To promote and facilitate application of the extract to food, we also evaluated antimicrobial effects of rice hull smoke extract (RHSE) against *Salmonella* in laboratory media and in infected mice.[15] The in vitro results showed that antimicrobial effectiveness of the extract approaches that of the widely used medicinal antibiotic vancomycin. In vivo, the observed reduction in pathogen levels was accompanied by reduction in NO levels produced by the peritoneal macrophages and an increase in the levels of recombinant interferon-γ (rIFN-γ) from splenocytes. The observed change in the latter biomarker implies that the extract also stimulated the cellular immune system. The extract also protected mice against *Salmonella*-induced liver necrosis and mortality. These beneficial effects suggest that RHSE is a novel bioactive formulation that merits

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further study for its potential to improve microbial food safety and human health.

The main objective of the present study was to evaluate the potential of the extract to reverse adverse consequences of alloxan-induced diabetes in mice. To our knowledge, this is the first report on the antidiabetic properties and associated molecular, cellular, and genetic events of a rice hull smoke extract.

MATERIALS AND METHODS

Materials. RPMI 1640 medium, fetal bovine serum (FBS), and other miscellaneous cell culture reagents were purchased from Hyclone Laboratories (Logan, UT). 2,7-Dichlorofluorescein diacetate (DCF-DA) and 4,5-diaminofluorescein diacetate (DAF-2/DA) were purchased from Sigma-Aldrich (St. Louis, MO) and Cayman Chemical (Ann Arbor, MI), respectively. Alloxan, NADH, NADPH, hematoxylin, eosin Y, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), glucose-6-phosphatase dehydrogenase, phosphoenolpyruvate (PEP), malic dehydrogenase, and other reagents of analytical grade were purchased from Sigma-Aldrich. The AMV reverse transcriptase and dNTP mixture were obtained from Takara Bio (Kyoto, Japan). PCR primers, as shown in Table 1, were custom-synthesized and purified by Bioneer (Daejon, Korea).

Table 1. Primer Sets Representing Four Target Genes and the Internal Control β-Actin Gene

primer	sequence
inducible nitric oxide synthase (iNOS) sense	5′-ATGTCCGAAGCAAACATCAC-3′
iNOS antisense	5′-TAATGTCCAGGAAGTAGGTG-3′
glucose-6-phosphatase (G6 Pase) sense	5′-AAGACTCCCAGGACTGGTTCATCC-3′
G6 Pase antisense	5′-TAGCAGGTAGAATCCAAGCGCG-3′
glucokinase (GCK) sense	5′-TTCACCTTCTCCTTCCCTGTAAGGC-3′
GCK antisense	5′-TACCAGCTTGAGCAGCACAAGTCG-3′
phosphoenolpyruvate carboxykinase (PEPCK) sense	5′-TGCTGATCCTGGGCATAACTAACC-3′
PEPCK antisense	5′-TGGGTACTCCTTCTGGAGATTCCC-3′
β-actin sense	5′-GTGGGGCGCCCCAGGCACCA-3′
β-actin antisense	5′-GTCCTTAATGTCACGCACGATTTC-3′

Preparation of Rice Hull Smoke Extract. The industrial production of the rice hull liquid smoke, its content of 161 compounds characterized by GC-MS, and beneficial bioactivities in chemical, cell, and bacterial assays are described in our previous publications.[8,15]

Mammalian Cell Culture. INS-1 rat insulinoma pancreatic β-cells from the American Type Tissue Culture Collection (Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS containing 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

Measurement of Cellular Peroxide Scavenging. The scavenging of reactive oxygen species (ROS) by RHSE in a cell milieu was performed as previously described.[16] The intracellular peroxide level was determined using a nonfluorescence probe, DCF-DA, which emits fluorescence following oxidation by hydrogen peroxide produced during an oxidative respiratory burst. Rat insulinoma INS-1 cells were seeded into a 24-well plate at a density of 5×10^5 cells/well and cultured in serum-free RPMI 1640 medium with low glucose for 24 h. Then, following the addition of 20 μM DCF-DA, incubation continued for 30 min in the dark at 37 °C. The DCF-DA-loaded cells were washed with phosphate-buffered saline (PBS, pH 7.4) and were then incubated for 1 h with the addition of 10 mM alloxan plus rice hull smoke extracts (final 0.1 or 0.5%, v/v). At the end of the incubation, the cells were lysed with 1 N NaOH, and aliquots (200 μL) of the cell lysate were transferred to a 96-well black plate. The fluorescence generated by ROS was monitored using a fluorescence plate reader (model Gemini-EM, Molecular Device, Sunnyvale, CA) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The results are expressed as fluorescence intensity (arbitrary units).

Cell Viability Assay. Cell viability was assessed by MTT staining as previously described.[17] Briefly, the INS-1 cells were seeded into a 96-well plate at a density of 5×10^5 cells/well and cultured for 24 h at 37 °C humidified air with 5% CO_2. The cells were then subjected to treatment with 0.1 or 0.5% RHSE for 48 h. After treatment, cells were stained with the addition of MTT. The resultant intracellular chromogen formazan product was solubilized by adding DMSO. Absorbance of the chromogen was read in a microplate reader (model 550, Bio-Rad, Hercules, CA) at 570 nm and a reference wavelength of 655 nm. Cell viability was expressed as a percentage of live cells relative to that of a normal control group treated with a vehicle (PBS) alone.

Mouse Feeding Study. Pathogen-free female ICR mice (6 weeks old) were purchased from Orient Bio (Seoul, Korea). After acclimation for 1 week, the mice were housed in a stainless steel cage under a 12 h light/dark cycle with a temperature range of 20−22 °C and relative humidity of 50 ± 10% and fed pelletized commercial chow diet without or with 0.5 or 1% RHSE (v/w) and sterile tap water ad libitum for 2 weeks (n = 10). Mice were then injected intraperitoneally with alloxan (100 mg/kg). Feeding continued during the entire experimental period. At the end of the experimental period, the mice were sacrificed by CO_2 inhalation. Blood samples were collected by cardiac puncture. To obtain the serum, microcentrifugation was carried out at 13000 rpm for 15 min. The liver and pancreas were excised, rinsed with PBS, and stored at −70 °C until analysis.

Glutamate Oxaloacetate (GOT) and Glutamate Pyruvate Transaminase (GPT) Assays. Serum enzyme GOT/GTP concentrations were determined using a colorimetric kit (Asan Pharmaceutical, Seoul, Korea) following the manufacturer's protocol. Briefly, diluted serum (20 μL) was added to the reaction solution. The resultant mixture was incubated at 37 °C for 30 and 60 min for GOT and GTP, respectively. The absorbance of the solution was determined at 505 nm using a microplate reader.

Nitric Oxide (NO) Generation. NO generation in living cells was monitored using a membrane-permeable fluorescent indicator DAF-2/DA as previously described,[18] with some modification. Briefly, INS-1 cells were seeded into 24-well plate at a density of 1×10^5 cells/well and cultured in serum-free RPMI 1640 medium with low glucose at 37 °C for 24 h. Cells were then incubated with 2 nmol/L DAF-2/DA at 37 °C for 30 min in the dark. DAF-2-loaded cells were treated with alloxan (10 mM) and/or the diluted rice hull smoke extracts for 6 h. At the end of the incubation period, the cells were lysed with 1 N NaOH (1 mL). Aliquots (200 μL) of the cell lysates were transferred to a 96-well black plate. The fluorescence generated by NO was monitored by a fluorescence plate reader at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The results are expressed as fluorescence intensity (arbitrary units).

Blood Glucose Level Assay. Blood samples were drawn from the tail vein of the mice 3−7 days after intraperitoneal (ip) injection of alloxan. The blood glucose level in mice was measured using the Accu-Chek Active kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction.

Glycogen and Insulin Levels of the Liver. The glycogen concentration in liver was measured as previously described.[19] Freshly excised liver (100 mg) was mixed with KOH (30%) and heated at 100

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°C for 30 min. The mixture was then added to ethanol (1.5 mL, 95%) and kept overnight at 4 °C. The pellet was mixed with distilled water (4 mL). An aliquot (500 μL) of the mixture was added to anthrone (0.2% in 95% H_2SO_4). The absorbance of the sample solution was then measured at 620 nm. The calculated results are based on a standard glucose calibration curve. The insulin content of the INS-1 cell culture supernatant and the mouse serum were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Billerica, MA).

Hepatic Glucose-Regulating Enzyme Activities. The hepatic enzyme source was prepared as previously described.[20] The glucokinase (GCK) activity was determined as previously described,[21] with slight modification. The reaction mixture (98 μL) containing the following ingredients was preincubated at 37 °C for 10 min: Hepes−NaOH (50 mM, pH 7.4), KCl (100 mM), $MgCl_2$ (7.5 mM), dithioerythritol (2.5 mM), albumin (10 mg/mL), glucose (10 mM), glucose-6-phosphate (G6 Pase) dehydrogenase (4 units), NADH (50 mM), and cytosol (10 μL). The reaction was initiated with the addition of ATP (10 μL of 5 mM) followed by incubation at 37 °C for 10 min. The change in absorbance at 340 nm was recorded.

The G6 Pase activity was measured following the method of Alegre et al.[22] The reaction mixture contained Hepes−NaOH (765 μL of 131.58 mM; pH 6.5), EDTA (100 μL of 18 mM; pH 6.5), G6 Pase (100 μL of 265 mM), NADPH (10 μL of 0.2 M), mutarotase (0.6 IU/mL), and glucose dehydrogenase (0.6 IU/mL). After preincubation at 37 °C for 3 min, the mixture was added to microsomes (5 μL) and incubated at 37 °C for 4 min. The change in absorbance at 340 nm was also recorded. The phosphoenolpyruvate carboxykinase (PEPCK) activity was determined following the method of Bentle and Lardy.[23] Briefly, the reaction mixture (1 mL) containing the following ingredients was incubated at 25 °C for 1 h: sodium Hepes (72.92 mM; pH 7.0), dithiothreitol (10 mM), $NaHCO_3$ (500 mM), $MnCl_2$ (10 mM), NADH (25 mM), IDP (100 mM), PEP (200 mM), malic dehydrogenase (7.2 units), and cytosol (10 μL). Enzyme activity was based on the decrease in the absorbance of the mixture at 350 nm at 25 °C.

Histopathology of Pancreas and Liver Tissues. For histological analysis, the liver and pancreas tissues were fixed with 4% paraformaldehyde in 0.5 M phosphate buffer (pH 7.4). The tissues were then rinsed with water, dehydrated with ethanol, and embedded in paraffin. The samples were sectioned into 4 μm and mounted onto glass slides. The sections were dewaxed using xylene and ethanol and then stained with hematoxylin and eosin Y (H&E) to reveal the hemorrhagic necrosis in the liver or the Langerhans islet of the spleen. Each lesion was counted in six blindly chosen random fields under the microscope at 100× magnification, and the incidence rate was recorded.

Reverse Transcription (RT) PCR of Cellular RNA. Total cellular RNA was prepared from liver tissue following acid phenol guanidium thiocyanate−chloroform extraction.[24] For RT, total RNA (1 μg) was incubated with AMV reverse transcriptase (5 U) and oligo (dT18) as primer (100 ng), except that a 6-mer random primer (IDT, Coralville, IA) was used for the reverse transcription of the PEPCK gene. DNA amplification was then primed in a reaction mixture containing dNTP mix (400 μM), *Taq* polymerase (2.5 U), and primer sets (20 μM each) representing target genes. PCR was conducted using a thermocycler (model PTC-200, MJ Research Inc., Reno, NV) with one cycle for 5 min at 94 °C, followed by 30 cycles for 30 s at 94 °C, 45 s at 58 °C, and 45 s at 72 °C, and finally one cycle for 5 min at 72 °C. All amplified PCR products were subjected to 1.5% agarose gel electrophoresis and visualized with a UV illuminator. The intensity of separated bands under DNA was quantified using a gel documentation system (model LAS-1000CH, Fuji Photo Film Co., Tokyo, Japan).

Western Blot Analysis of INS-1 Cell Proteins. INS-1 cells were lysed and extracted with RIPA buffer (50 mM Tris Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, pH 7.4). Protein concentrations were determined according to the Bradford method using a Bio-Rad Protein Kit (Hercules, CA). Bovine serum albumin (BSA) was used as standard. Tissue extracts containing proteins (30 μg) were separated on 10% polyacrylamide gels and electrophoretically transferred onto a nitrocellulose membrane (Millipore, Billerica, MA). The rabbit anti-rat iNOS polyclonal antibody (Cell Signaling Tec., Danvers, MA) and anti-rat β-actin monoclonal antibody (Millipore) were used as probes for Western blot analysis. After blocking with 5% skim milk, membranes were incubated with each primary antibody, followed by HRP-conjugated anti-IgG antibodies. Blots were developed using the ECL detection kit (Pierce, Rockford, IL). The intensity of separated protein bands was quantified using a gel documentation system (model LAS-1000CH, Fuji Photo Film Co.). At least three separate replicates were determined for each experiment.

Statistical Analysis. Results are expressed as the mean ± SD of three independent experiments. Significant differences between means were determined using the Statistical Analysis software package (SAS, Cary, NC). $p < 0.05$ is regarded as statistically significant.

RESULTS AND DISCUSSION

Effect of RHSE on Oxidative Stress and Cell Death Induced by Alloxan in Vitro. The inhibitory effects of rice hull smoke extract on oxidative stress and cell death were measured in rat insulinoma β-cell line INS-1, widely used in studies of the mechanism of diabetes prevention.[24,25] Compared to the alloxan-treated control, the 0.1 and 0.5% RHSE treatments suppressed intracellular peroxide levels by about 42 and 58%, respectively (Figure 1). A dose-dependent

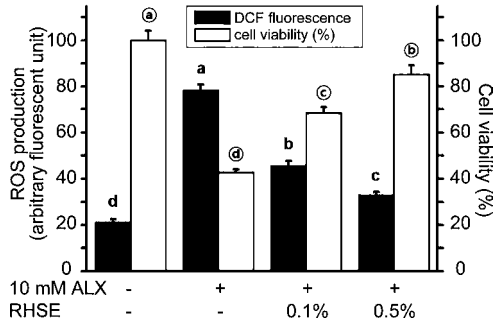

Figure 1. Inhibitory effects of RHSE on alloxan-induced ROS generation and cytotoxicity in INS-1 cells. The intracellular peroxide level is expressed as an arbitrary unit of the intensity of fluorescence emitted from the oxidation product of DCF-DA. Data are expressed as the mean ± SD ($n = 3$). Bars not sharing a common letter are not significantly different between groups at $p < 0.05$.

decrease in the intracellular oxidative level was found to be inversely related to cell viability. Compared to the alloxan-treated control group, the 0.1 and 0.5% RHSE treatments increased cell viabilities by about 57 and 95%, respectively. These observations suggest that rice hull extract prevented pancreatic β-cell death due to oxidative damage.

Effect of RHSE on NO Production in Vitro. Because NO is known to react with the superoxide anion to produce the highly toxic peroxynitrite radical, experiments were performed to find out whether alloxan can induce NO production in INS-1 cells. DAF-1/DA was used to probe intracellular NO production levels because nonfluorescent DAF-2/DA specifically reacts with NO to produce fluorescent DAF-2. As shown in Figure 2, RHSE inhibited NO production in INS-1 cells in a dose-dependent manner. Compared to the alloxan-treated control, treatments with 0.1 and 0.5% RHSE induced 13 and 43% inhibitions, respectively. RT-PCR and Western blot analysis showed that the decrease in NO production by

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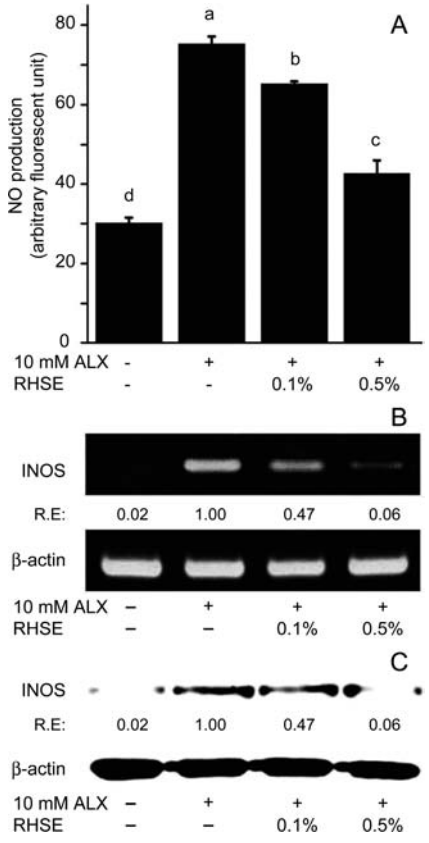

Figure 2. Inhibitory effects of RHSE on NO production in the alloxan-stimulated INS-1 cells. (A) RHSE treatment modulates alloxan-induced intracellular NO production in INS-1 cells. The intracellular NO level is expressed as an arbitrary unit of the intensity of fluorescence emitted from oxidation of DAF-2/DA. Data are expressed as the mean ± SD (n = 3). Bars not sharing a common letter are not significantly different between groups at $p < 0.05$. (B) The modulatory effect of RHSE on iNOS gene expression was assessed by semiquantitative RT-PCR. (C) RHSE-modulated iNOS protein expression in alloxan-stimulated INS-1 cells was analyzed by Wester blot. The relative ratio of expression of each gene or protein in INS-1 cells is expressed as the relative expression (RE) value calculated from target gene/β-actin gene expression. Figures represent results from at least three individual experiments.

RHSE mainly resulted from transcriptional down-regulation of iNOS gene expression.

Anti-inflammatory Activity of RHSE Resulting from Inhibition of Pro-inflammatory Cytokine Expression and Restoration of Insulin Release. To find out whether inflammatory damage of β-cells could be ameliorated by RHSE treatment, changes in the expression profiles of the pro-inflammatory cytokine genes IL-1β, IL-6, and TNF-α were assessed by RT-PCR in INS-1 cells (Figure 3). Compared to the alloxan-treated control, the 0.5% RHSE treatment suppressed in a dose-dependent manner the alloxan-induced transcription of these pro-inflammatory cytokine genes by about 70, 63, and 88%, respectively. It seems that RHSE can ameliorate the incidence of diabetes through suppression of inflammation-triggered β-cell injury. This suggestion is supported by the observation that marked suppression of insulin secretion induced by alloxan was reversed by RHSE treatment. Table 2 shows 19 and 37% increases in insulin secretion induced by 0.5 and 1% RHSE, respectively.

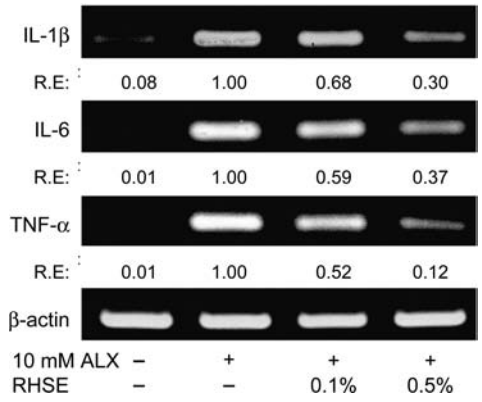

Figure 3. Semiquantitative RT-PCR analysis of RHSE-modulated gene expressions for synthesis of pro-inflammatory cytokines in the alloxan-stimulated INS-1 cells. The relative ratio of expression of each gene in INS-1 cells is expressed as a relative expression (RE) value calculated from target gene/β-actin gene expression. Figures represent results from at least three individual experiments.

Table 2. Effect of RHSE on Insulin Release from Alloxan-Treated INS-1 Cells[a]

expt	insulin release,[a] ng/mL (% secretion)
normal control (PBS)	1.663 ± 0.068 (100)
10 mM alloxan-treated control	0.844 ± 0.056 d (50.75)
10 mM alloxan + 0.5% RHSE	1.154 ± 0.113 c (69.37)
10 mM alloxan + 1% RHSE	1.463 ± 0.126 b (87.99)

[a]Data are expressed as the mean ± SD (n = 3). Values in each column with the same letter are not significantly different between groups at $p < 0.05$.

RHSE Restores Blood Glucose, Serum Insulin, and Hepatic Glycogen Levels in Alloxan-Induced Diabetic Mice. As noted above, RHSE protected the β-INS-1 cells against local inflammatory damage triggered by oxidative stress. It was, therefore, of interest to find out whether dietary administration of RHSE could protect pancreatic β-cells against oxidative damage in an alloxan-induced type 1 diabetic mouse model (Table 3). The initial blood glucose, serum insulin, and hepatic glycogen levels in mice did not significantly differ among the groups (data not shown). However, the alloxan treatment induced a significant increase in the glucose level. In contrast, compared to the alloxan-treated control group, mice fed 0.5 and 1% RHSE exhibited 22 and 45% lower blood glucose levels, respectively. The alloxan treatment also resulted in a significant 81% decrease in serum insulin level compared with normal control mice. As expected, the dietary administration of 0.5 and 1% RHSE increased serum insulin levels about 2- and 3-fold, respectively. With 1% RHSE, the serum insulin level was restored to 82% of that found in the control mice. Administration of 0.5 and 1% RHSE restored glycogen content in the liver to 95% of the levels in the control mice. These results indicate that dietary administration of rice hull smoke extract ameliorate incidence of type 1 diabetes in alloxan-treated mice, possibly by preventing damage to the β-cells of the pancreas.

RHSE Inhibits Alloxan-Induced Liver Injury. As shown in Figure 4, the alloxan-treated control group exhibited markedly elevated concentrations of GOT and GTP, indicating the induction of severe liver damage. RHSE administration markedly lowered serum levels of these enzymes. With 1%

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Table 3. Effect RHSE on Blood Glucose, Serum Insulin, and Hepatic Glycogen Levels in Alloxan-Induced Diabetic Mice[a]

expt	blood glucose, mg/dL	serum insulin, ng/mg protein	glycogen, mg/g liver wt
normal control (PBS)	134.400 ± 6.309 d	63.714 ± 3.050 a	3.279 ± 0.226 a
diabetic control (alloxan)	300.400 ± 16.087 a	12.345 ± 0.882 d	2.648 ± 0.151 c
alloxan + 0.5% RHSE	234.000 ± 25.875 b	37.893 ± 2.013 c	2.922 ± 0.147 b
alloxan + 1% RHSE	166.000 ± 14.866 c	52.005 ± 3.915 b	3.165 ± 0.021 ab

[a]Data are expressed as the mean ± SD (n = 3). Values in each column with the same letter are not significantly different between groups at $p < 0.05$.

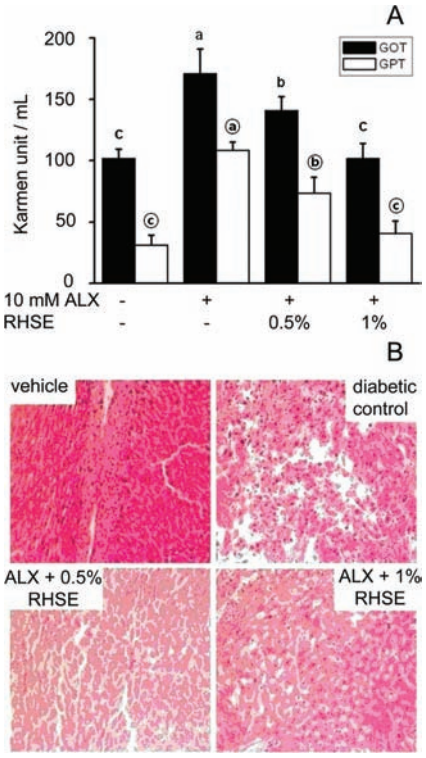

Figure 4. Modulation of alloxan (ALX)-induced liver injury by RHSE. (A) Dietary RHSE suppresses serum glutamic oxaloacetic transaminase/glutamic pyruvic transaminase (GOT/GPT) activities. Data are expressed as the mean ± SD (n = 10). Bars not sharing a common letter are not significantly different between groups at $p < 0.05$. (B) RHSE ameliorates liver damage induced by alloxan in a dose-dependent manner. Each liver specimen was fixed with 4% paraformaldehyde, and sections were stained with hematoxylin and eosin (H&E). Magnification, ×100. Figures represent results from at least three individual experiments.

RHSE, GOT and GTP levels were restored to almost the same level as that of the normal control group. Histology revealed extensive liver injuries such as necrosis and hemorrhage in the alloxan-treated diabetic control mice. By contrast, minimal liver damage was observed in mice orally administered 0.5 and 1% RHSE (Figure 4).

RHSE Restores Hepatic Glucose-Regulating Enzyme Activities. Table 4 shows that compared with the alloxan-treated diabetic control group, the hepatic GCK activity was 27 and 62% higher in mice fed 0.5 and 1% RHSE, respectively. Compared with the vehicle-treated control, the alloxan-treated diabetic group also showed a marked 54% increase in G6 Pase activity. By contrast, compared with the alloxan-treated diabetic control group, the dietary administration of RHSE suppressed the rise of the enzyme activity to normal levels by 20 and 45%, respectively. Similarly, compared with alloxan-treated diabetic control group, 13 and 29% decreases of PEPCK activities were observed in mice fed 0.5 and 1% rice RHSE-supplemented diets, respectively. RT-PCR analysis indicates that an alteration in these hepatic glucose-regulating enzyme activities was closely associated with the extent of expression of associated genes (Figure 5).

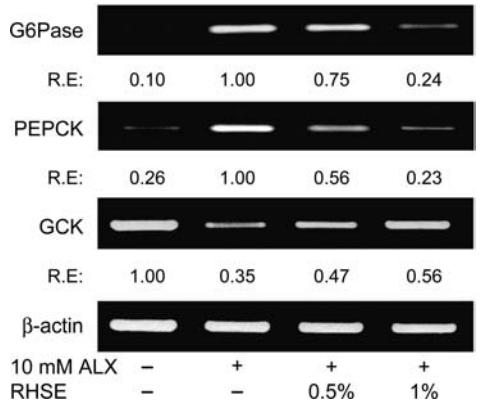

Figure 5. Semiquantitative RT-PCR analysis of RHSE-modulated gene expressions for the synthesis of representative enzymes involved in hepatic glucose metabolism in the alloxan-stimulated INS-1 cells. The relative ratio of expression of each gene in INS-1 cells is expressed as a relative expression (RE) value calculated from target gene/β-actin gene expression. G6 Pase, PEPCK, and GCK represent glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, and glucokinase, respectively. Figures represent results from at least three individual experiments.

RHSE Restores Alloxan-Induced Damage of Pancreatic Langerhans Islet Cells. Histopathology of tissues shows

Table 4. Effect of RSHE on Hepatic G6 Pase, PEPCK, and GCK Enzyme Activities in Alloxan-Induced Diabetic Mice[a]

	enzyme activities, nmol/min/mg protein		
expt	G6 Pase	PEPCK	GCK
normal control (PBS)	79.031 ± 1.667 d	21.135 ± 2.311 d	11.313 ± 0.755 a
diabetic control (alloxan)	161.851 ± 4.465 a	32.576 ± 1.245 a	6.174 ± 0.551 d
alloxan + 0.5% RHSE	129.036 ± 4.308 b	28.334 ± 1.457 b	7.850 ± 0.327 c
alloxan + 1% RHSE	88.296 ± 4.362 c	23.221 ± 2.198 c	9.983 ± 0.486 b

[a]Data are expressed as the mean ± SD (n = 3). Values in each column with the same letter are not significantly different between groups at $p < 0.05$.

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normal acini and normal cellular population in the Langerhans islet of the pancreas (Figure 6). By contrast, compared to the

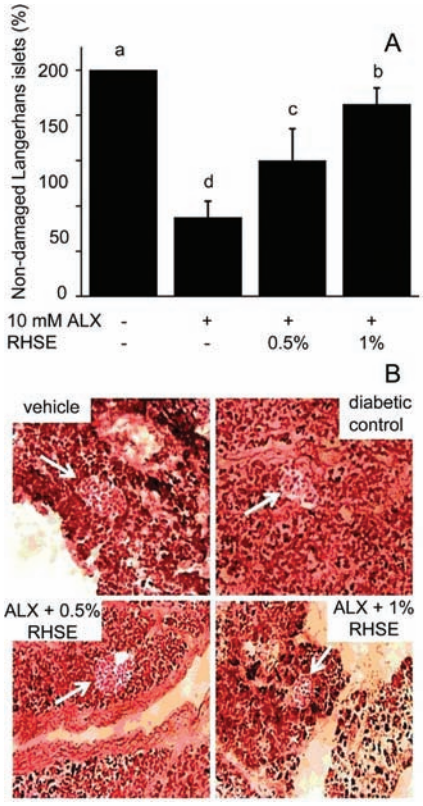

Figure 6. Inhibitory effect of RHSE on alloxan-induced atrophy of Langerhans islet of the pancreas. (A) Intact Langerhans islets were counted under a microscope in six randomly chosen fields. Results are expressed as the mean ± SD (n = 10). Bars not sharing a common letter are not significantly different between groups at $p < 0.05$ compared with the vehicle value. (B) To observe blood-vessel formation, paraformaldehyde-fixed and paraffin-embedded tumor sections were stained with hematoxylin and eosin Y (H&E). The images were photographed by microscope at 100× magnification.

control mice, the alloxan treatment induced a 75% reduction in size and damage to the Langerhans islet. The size of the islet and the cell population were restored to normal levels following RHSE treatment.

Related Previous Studies. To place the findings of the present study in proper perspective and to stimulate studies on possible additive or synergistic antidiabetic effects of RHSE with other antidiabetic plant-derived substances, we will briefly mention several reported studies on the antidiabetic potential of food ingredients, herbal and medicinal plant extracts, and isolated compounds. Rice bran and constituents, some of which are present in rice hull smoke,[8] exhibit antidiabetic effects in humans and rodents.[26−28] Mushrooms are a source of antidiabetic polysaccharides.[29] Two characterized tetrasaccharide glyceroglycolipids isolated from pumpkin fruit (*Curcubita moschata*) induced significant glucose-lowering effects in streptozotocin- and high-fat-diet-induced diabetic mice.[30] Continuous administration for 3 weeks of a polysaccharide from tea (*Camellia sinensis*) inhibited α-amylase and α-glucosidase in vitro and reduced blood glucose levels of alloxan-induced diabetic mice.[31] Scoparic acid D isolated from the Indian plant *Scoparia dulcis* protected male Wistar rats against streptozotocin-induced hyperglycemia, oxidative stress, and damage of pancreatic β-cells associated with pathogenesis of diabetic complications.[32] Butyl isobutylphthalate isolated from the *Laminaria japonica* rhizoid plant inhibited α-glucosidase in vitro (IC_{50} = 38 μM) and induced significant hypoglycemia in streptozotocin-induced diabetic mice.[33] An extract of the Indian plant *Butea monosperma* exhibited hypoglycemic and antioxidant activity in alloxan-induced mice.[34] The antioxidative and antihyperglycemic effects of lemon balm (*Melissa officinalis*) essential oil in type 2 diabetic mice resulted from the inhibition of glucose- and lipid-regulating enzymes and enhanced glucose uptake and metabolism in the liver.[35] An extract of the edible plant *Opuntia milpa* Alta significantly decreased blood glucose levels in streptozotocin-induced diabetic mice.[36] The antihyperglycemic mechanism of extracts of aerial parts of the Nigerian herb *Phylanthus nirui* in alloxan diabetic rats seems to be due to inhibition of α-amylase and α-glucosidase regulating enzymes.[37] An aqueous extract of the Gabon plant *Tabernanhte iboga* induced insulin secretion in rat pancreatic islets and increased insulin secretion by medicinal drugs.[38] Aqueous extracts of several medicinal plants grown in Nigeria exhibited antidiabetic effects in alloxan-induced diabetic mice.[39] The extract from *Anisopus manii* had the greatest effect.

Dietary Significance. The results of the present study with the liquid rice hull extract complement and extend the above-cited findings on the antidiabetic potential of plant extracts and some of their bioactive constituents. An unanswered question is whether the antidiabetic effects of RHSE are caused by individual or combinations of compounds acting additively or synergistically. This aspect merits further study.

Taken together, the described findings from biomarker, cell viability, cytokine gene expression, enzyme, histology, oral feeding, and reactive oxygen assays indicate that the protective effect of RHSE against diabetes in the mouse can be attributed to blockage of oxidative stress-induced damage of Langerhans islet β-cells of the pancreas and improved metabolism of glucose in the liver. The results imply that rice hull extract-supplemented functional foods may contribute to the prevention and management of diabetes. Because the previous and present studies show that the extract also exhibited anti-inflammatory and antimicrobial properties, rice hull smoke-treated foods may have advantages over the widely used wood smoke treatments designed to impart flavoring and preservative effects.[40−42] The >100 million tons of rice hulls produced worldwide provide a new source of bioactive compounds derived from an agricultural byproduct that merit further evaluation for their potential to impart health-promoting effects to food.

Abbreviations Used

BSA, bovine serum albumin; DCF-DA, dichlorofluorescein diacetate; DAF2/DA, diaminofluorescein diacetate; dNTP, 2′-deoxyribonucleoside triphosphate; ELISA, enzyme-linked immunosorbent assay; FPS, fetal bovine serum; GC-MS, gas chromatography−mass spectrometry; GCK, glucokinase; GOT, glutamate oxoloacetate; G6 Pase, glucose-6-phosphatase; GPT, glutamate pyruvate transaminase; IDP, inosine 5′-diphosphate; IL-1β, interleukin-1β; IL-6, interleukin-6; INS-1, insulinoma-1 cell line; iNOS, inducible nitric oxide synthase; ip, intraperitoneal; NO, nitric oxide; PBS, phosphate buffered saline; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; RE, relative expression of genes and proteins;

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rIFN-γ, recombinant interferon-γ; RHSE, rice hull smoke extract; ROS, reactive oxygen species; RT-PCR, reverse transcription−polymerase chain reaction; TNF-α, tumor necrosis factor-α.

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