Huang-Lian-Jie-Du-Tang Supplemented with Schisandra chinensis Baill. and Polygonatum odoratum Drue Improved Glucose Tolerance by Potentiating Insulinotropic Actions in Islets in 90% Pancreatectomized Diabetic Rats

Sunmin Park,1,1 Sang Mee Hong,1 II Sung Ahn,1 Yang Jin Kim,2 and Jung Bok Lee2

1Department of Food and Nutrition, College of Natural Science, Hoseo University, Asan-Si, 336-795, Korea
2Naturalbiopharms Co., Ltd., Seoul, 135-010, Korea

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We investigated to determine what effects, if any, the respective water extracts of Radix scutellariae (RS), Fructus schisandrace chinensis (FSC), Huang-Lian-Jie-Du-Tang (HLJDT), and HLJDT supplemented with FSC, and Rhizoma Polygonati odorati (HLJDT-M) would have on glucose tolerance by modulating glucose-stimulated insulin secretion, β-cell mass, and morphometry in 90% pancreatectomized (Px) diabetic rats fed high-fat diets. Through the elevation of intracellular cAMP levels, FSC RS, HLJDT, and HLJDT-M increased insulin secretion in Min6 cells and GLP-1 secretion in NCI-H716 cells. After an 8-week period of treatment, it was found that HLJDT-M improved glucose tolerance in an oral glucose tolerance test in Px rats. HLJDT-M also potentiated first- and second-phase insulin secretion, but RS and HLJDT elevated only the second phase at hyperglycemic clamp. RS and HLJDT increased β-cell mass by hyperplasia and hypertrophy, while HLJDT-M increased it only by hyperplasia. The rise in hyperplasia was associated with elevated IRS2 and PDX-1 expression in the islets. In conclusion, HLJDT-M worked as an anti-diabetic prescription by enhancing insulinotropic actions in diabetic rats.

Key words: glucose homeostasis; insulin secretion; β-cell mass; hyperplasia; hypertrophy

Type-2 diabetes is a heterogeneous metabolic disorder characterized by impairment of insulin secretion from pancreatic β-cells and insulin resistance in peripheral tissues such as skeletal muscle and adipose tissue, and in the liver.1,2 In most cases, insulin resistance precedes dysregulation of insulin secretion in humans.2,3) Since obesity induces insulin resistance in the liver, skeletal muscles, and adipose tissues, elevation of insulin secretion maintains normoglycemia by compensating for insulin resistance.1,2) Thus, as long as insulin secretion compensates for insulin resistance, diabetes does not develop.4,5) Along with enhanced glucose-stimulated insulin secretion, β-cell mass should be sustained for a long period.3,4) Hence, herbs and drugs for type-2 diabetes mellitus should have an insulin-sensitizing action to attenuate insulin resistance, and an insulinotropic action to improve glucose-stimulated insulin secretion and pancreatic β-cell survival.

The first generation of type-2 diabetic drugs was based on insulin secretagogue such as sulfonylurea derivatives. Sulfonylureas effectively release insulin from pancreatic β-cells regardless of serum glucose levels by activating the sulfonylurea receptor to close ATP-sensitive potassium channels.5) It lowers serum glucose levels not only in hyperglycemic states but also in normoglycemic ones.6) As a result, sulfonylureas induce frequent hypoglycemia and cause exhaustion of and damage to β-cells, which leads to an insufficient release of insulin when serum glucose levels are increased.5,6) Eventually, sulfonylurea derivatives can exacerbate the symptoms of diabetes.7) The ideal insulin secretagogue should induce acute-phase insulin secretion sufficiently during hyperglycemia, and should increase pancreatic β-cell mass by potentiating β-cell proliferation and reducing β-cell apoptosis. Hence, an insulinotropic agent that enhances glucose-stimulated insulin secretion and enlarges β-cell mass is a better anti-diabetic agent than a simple insulin secretagogue that increases insulin release regardless of the glucose concentration. A recent study reported that a glucagon-like peptide-1 (GLP-1) enhances insulinotropic actions and thus relieves type-2 diabetic symptoms.8,9) GLP-1 is secreted from L-cells in the intestines and works through the GLP-1 receptor in the membranes of various cells, such as the neurons, pancreatic β-cells, and hepatocytes.8,9) It activates adenyl cyclase to increase intracellular cAMP, and further cascading pathways are potentiated.8) However, GLP-1 is quickly degraded by dipeptidyl peptidase IV (DPP-IV).10,11) Recently, a GLP-1 receptor agonist, exenatide, and DDP-IV inhibitors, have been approved by the US Food and Drug Administration for treating type-2 diabetic patients.10,11) Exenatide improves glucose-stimulated insulin secretion through the expansion of β-cell mass by inducing insulin receptor substrate-2 (IRS2) expression in β-cells.9,10,12) In addition, there is some evidence that exenatide enhances peripheral insulin sensitivity.13,14)

Our previous in vitro study revealed that the berberine contained in Rhizoma coptidis (Coptis chinensis Franch.) improves insulin sensitizing and insulinotropic actions.15) Berberine is also reported to potentiate the activity and phosphorylation of AMPK, which enhances glucose uptake in skeletal muscles and the liver.16)
Furthermore, our preliminary study revealed that Radix scutellariae (Scutellaria baicalensis Georgi; RS), and Cortex phellobindi (Phellobodium amurense Rupr.) enhanced glucose-stimulated insulin secretion in Min6 cells. Among well-known herbal prescriptions, there is Huang-lian-je-du-tang which includes Rhizoma coptidis, RS, Cortex phellobindi (HLJDT; Korean name, Hwang-reon-hae-dok-tang), and Fructus gardenia (Gardenia jasminoides Ellis.). HLJDT is a traditional Chinese medicine prescription known to have properties to protect the liver, including hepatic detoxification and to improve hepatotoxin-induced liver injuries.17) The name, HLJDT, originated from its components and its function. Although HLJDT sufficiently increased glucose-stimulated insulin secretion in Min6 cells, it did not improve insulin-stimulated glucose uptake in 3T3-L1 adipocytes in our preliminary study. This suggests that HLJDT did not have an insulin sensitizing property. Thus, since water extracts of Fructus schisandrae chinensis (Schisandra chinensis Baill.; FSC) and Rhizoma Polygonati odorati (Polygonatum odoratum Druce) were found to reduce insulin resistance in several in vitro and in vivo studies, including our own, FSC and Rhizoma Polygonati odorati were added to HLJDT to give it insulin sensitizing properties (HLJDT-M).18-20) Hence we tried to determine whether RS, FSC, HLJDT, and HLJDT-M would improve glucose tolerance in 90% pancreatectomized diabetic rats by enhancing insulinotropic actions.

Materials and Methods

Materials. The HLJDT prescription consisted of equal amounts of RS, Cortex phellobindi, Rhizoma coptidis, and Fructus gardenia, while the HLJDT-M prescription contained FSC, Rhizoma Polygonati odorati, Rhizoma coptidis, RS, Cortex phellobindi, and Fructus gardenia at a ratio of 2:2:1:1:1:1. All of these herbs were purchased from the Kyung Dong Herb Market (Seoul, Korea), and were carefully authenticated by the Department of Quality Inspection and Examination of the Korea Institute of Oriental Medicine (Daejeon, Korea). When making the extract of HLJDT, RS, Cortex phellobindi, Rhizoma coptidis, and Fructus gardenia were mixed a given ratios, to make a total weight of 1,000 g. To make the extract of HLJDT-M, FSC, Rhizoma Polygonati odorati, Rhizoma coptidis, RS and Cortex phellobindi, and Fructus gardenia were mixed a given ratios to make a total weight of 1,000 g. Each mixture was decocted twice by refluxing the mixture in 4 l of HCl (0.1 N, 10 min) and vortexed. The suspension was homogenized and then centrifuged at 4°C for 1 h, and each extract was precipitated by centrifugation (1 min, 10,000 g, at 4°C). The extracts were stored at -80°C in appropriate volumes.

Glucose-stimulated insulin secretion in Min6 cells and GLP-1 secretion in NCI-H716 cells. Min6 cells of the insulinoma cell line were grown in high-glucose DMEM containing 15% vol/vol heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 50 μM β-mercaptoethanol (Sigma, St. Louis, MO), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma). The cells were used when passages 19 and 30 were maintained at 37°C in a humidified atmosphere with 5% CO2. Min6 cells at 70-80% confluence were used for glucose-stimulated insulin secretion. Min6 cells in a 24-well plate were washed and incubated with low-glucose DMEM with 0.5% wt/vol bovine serum albumin (BSA) for 8 h, and the media was changed to the same media containing 20 μg/ml water extracts of herbs and prescriptions or 2.5 nm exendin-4 (Sigma) for 8 h. At the end of the experiment, the media were switched to a KRB solution with low-glucose (2 mm) and 20 μg/ml water extracts or 2.5 nm exendin-4, and were incubated for 30 min, and consecutively incubated with a new KRB solution containing high-glucose (20 mm) and 20 μg/ml water extracts or 2.5 nm exendin-4 for 30 min. Exendin-4 (a commercial form of GLP-1 receptor agonist) was used as a positive control. The supernatant was collected by centrifugation (10 min, 10,000 g) and stored at -80°C for subsequent analysis. The cell media and homogenates were sonicated in a homogenization buffer. The buffer consisted of 1 N HCl containing 5% vol/vol HCOOH, 1% vol/vol trifluoroacetic acid, and 1% wt/vol NaCl. To normalize the GLP-1 content, cell homogenates were measured using the Bio-Rad protein assay kit. Ketones were extracted from the cell media and homogenates using an alcohol extraction method, as described by the supplier of the GLP-1 (7–36) Total RIA Kit (Linco Research, St. Charles, MO).

Animal procedures: pancreatectomy, high-fat diet, and medicine administration. Male Sprague Dawley rats, weighing 195 ± 16 g, were housed individually in stainless steel cages in a temperature- and humidity-controlled environment (23°C, 60%) on a normal 12 h light-dark cycle. All surgical and experimental procedures were performed in accordance with the recommendations found in the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health, USA, and were approved by the Institutional Animal Care and Use Committee of Hoseo University. To generate mild type-2 diabetic experimental animals, 90% of the pancreas was removed by the Hosokawa technique.21) After the 90% pancreatectomy (Px), the remaining pancreas was within 2 mm of the common bile duct and extended from the duct to the first part of the duodenum, and the weight of the remainder was 10 ± 2% of the total pancreas weight at the end of the Px procedure. Fewer than 10% of the rats died after the operation, and the surviving Px rats recovered from surgery within a week. Since the kidney threshold for glucose is about 9.4–10 mm,22) a serum glucose concentration of 9.4 mm at a random fed state was chosen as the cut-off parameter in this animal study. In addition, this was deemed a rational and appropriate cut-off point in order to exclude rats that do not develop type-2 diabetes after Px surgery as happened in previous studies, including our own.21,22,24) The Px rats included in the experiments exhibited the characteristics of mild and non-obese type-2 diabetes with insulin deficiency and insulin resistance. Since the Px rats had only approximately 50% insulin secretion capacity as compared to the sham-operated (Sham) rats at the hyperglycemic clamp, they developed hyperglycemia.23,24) Prolonged insulin deficiency developed into insulin resistance. Our previous study showed that Px rats increased insulin resistance by about 40–50%, as determined by the euglycemic hyperinsulimic clamp procedure.23,24) By contrast, the Sham rats had normal glucose homeostasis and did not show any diabetic symptoms. Since we characterized Px rats in comparison with Sham rats in our previous studies,23,24) we did not include Sham rats as a normal control group in the present study. As was found in our previous studies, the Px rats fed a high-fat diet increased in intracellular fat in various tissues, such as the liver, skeletal muscles, and adipose tissues. A high-fat diet exacerbates diabetic symptoms due to a heightening of insulin resistance and attenuation of β-cell function and mass.23,24) All the rats freely consumed water and a
high-fat diet during the 8-week experimental period. The high-fat diet consisted of 40 energy percent (Ear%) carbohydrates, 20 Ear% protein, and 40 Ear% fats, and was semi-purified by modifying the base with an AIN-93 formulation used in experimental diets.25) The major carbohydrate, protein, and fat sources were starch plus sugar, casein (milk protein) and shortening (CheiJeong, Seoul, Korea) respectively. The 9-week rats were randomly divided into four groups of 15 each with comparable mean body weights. The five different groups were orally supplemented with 200 mg of lyophilized water extracts of HLJDT, HLJDT-M, FSC, RS, or cellulose (a negative control) per kg of body weight on a daily basis. To compare the anti-diabetic effects of the remedies with a single herb, FSC was selected to evaluate its insulin sensitizing effects, and RS was chosen to evaluate its insulinotropic action.

Oral glucose tolerance test. Overnight fasting serum glucose levels, food and water intake, and body weights were measured every Tuesday at 10 AM. An oral glucose tolerance test (OGTT) was performed at the 6th week in the overnight fasted animals by orally administering 2 g of glucose/kg of body weight. Blood samples were taken by tail bleeding at 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 120 min after glucose loading, and serum glucose and insulin were measured with a Glucose Analyzer II (Beckman, Palo Alto, CA) and a radioimmunoassay kit (Linco Research, Billerica, MA) respectively. The average of the total areas under the curves for serum glucose and insulin were calculated by the trapezoidal rule. Since the baseline values of serum glucose and insulin were not significantly different among the groups, their baseline values were not considered in the calculation of the areas.

Hyperglycemic clamp. After 7 weeks of treatment, catheters were surgically implanted into the right carotid artery and left jugular vein of each rat in all groups after anesthetization with ketamine and xylazine had been done. Five to 6 d after implantation, hyperglycemic clamping was performed in freely moving overnight fasted rats to determine insulin secretion capacity, as described in previous reports.26,27) During the clamp, glucose was infused to maintain serum glucose levels at 5.5 mM above the baseline, and serum insulin levels were measured at designated times. Glucose infusion rates were calculated over 60–120 min to maintain hyperglycemic states. Insulin sensitivity at the hyperglycemic clamp was calculated as the ratio of the glucose infusion rate to steady-state plasma insulin levels.27) Insulin sensitivity indicated insulin sensitivity in a hyperglycemic state.

Sample collection. After clamping, the rats were freely provided food and water for 2 d. on the next day, they were deprived of food for 16 h. Nine to 10 rats from each group were injected with BrdU (100 μg/kg of body weight). Six h post-injection, the rats were anesthetized with intraperitoneal injections of ketamine and xylazine, and the pancreas was immediately dissected. The pancreas was fixed with 4% paraformaldehyde and paraffin-embedded, as described in our previous reports.12,28) The rest of the rats from each group (five rats per group) were used in isolating islet procedures. The islets were isolated with collagenase P (Roche Applied Science, Indianapolis, IN), as previously described.12) The islets were further used to assay intracellular cAMP levels and the mRNA levels of genes. The levels of cAMP were determined by the method described above for our in vitro study.

Immunohistochemistry and islet morphometry. Two serial 5-μm paraffin-embedded pancreas sections were selected out of seventh or eighth section to avoid counting the same islets twice when measuring the β-cell area. BrdU incorporation, and apoptosis. Endocrine β and α-cells were identified by applying guinea pig anti-insulin and rabbit anti-glucagon antibodies to the sections. BrdU incorporation in the β-cells was determined by staining rehydrated paraffin sections with anti-insulin and anti-BrdU antibodies.12,29) Apoptosis of β-cells was measured by a TUNEL kit (Roche Molecular Biochemicals, Indianapolis, IN) and counterstained with hematoxylin and eosin to visualize the islets.24) The pancreatic β-cell area was measured by examining all of the non-overlapping images in two insulin-stained sections of each rat at a magnification of 10 × with a Zeiss Axiosvert microscope (Carl Zeiss Microimaging, Thornwood, New York). The results of β-cell quantification were expressed as percentages of the total surveyed area containing insulin-positive cells, as measured with IP Lab Spectrum software (Scanalytics, Fairfax, VA). The pancreatic β-cell mass was calculated by multiplying the percentage of the insulin-positive area by the weight of the corresponding pancreatic portion.12,28,29) The individual β-cell sizes were determined as the insulin-positive area divided by the number of nuclei counted in the corresponding insulin-positive islets in randomly immunofluorescence-stained sections. An enlarged individual β-cell size indicates induction of β-cell hypertrophy.26) Beta-cell proliferation was calculated as the total number of BrdU+ nuclei in the β-cell nuclei per pancreas section.12,24) Apoptosis of β-cells was measured by the total number of apoptotic bodies in the β-cell nuclei per pancreas section.20)

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from the islets of five rats from each group using a monophasic solution of phenol and guanidine isothiocyanate (Trizol reagent, Gibco-BRL, Rockville, MD), followed by extraction and precipitation with isopropyl alcohol. The cDNA was synthesized from equal amounts of total RNA with superscript III reverse transcriptase, and the polymerase chain reaction (PCR) was performed with high-fidelity Taq DNA polymerase. Equal amounts of cDNA were mixed with sybergreen mix (Bio-Rad Laboratories), and these were analyzed with a realtime PCR machine (Bio-Rad Laboratories). The expression level of the gene of interest was corrected for that of the housekeeping gene, 18S. The primers used to detect rat IRS2 and IRS3 were designed to sandwich at least one intron to avoid amplification of contaminating genomic DNA.

Statistical analysis. Statistical analysis was performed using the SAS statistical analysis program, and all results were expressed as mean ± standard deviation. The anti-diabetic effects of HLJDT, HLJDT-M, FSC, and RS were determined by one-way analysis of variance. Significant differences in the main effects among groups were identified by the Tukey test among groups of equal sample sizes, or by the Tukey-Kramer test among groups of unequal sample sizes at p < 0.05.

Results

Glucose-stimulated insulin secretion in Min6 cells and GLP-1 secretion in NCI-H716 cells

We tested to determine whether the extract of FSC, Rhizoma Polygonati odorati, Rhizoma coptidis, RS, Cortex phellectronyi, Fructus gardenia, HLJDT, or HLJDT-M would improve glucose stimulated insulin secretion in Min6 cells and GLP-1 secretion in NCI-H716 cells. RS, HLJDT, and HLJDT-M increased glucose-stimulated insulin secretion more than the other treatments in the Min6 cells (Fig. 1A). Cortex phellodendri and Rhizoma coptidis also elevated secretion more than the DMSO treatment (control) in them (Fig. 1A). Exendin-4 (2.5 nm) released insulin more than HLJDT-M (p < 0.005). This was positively related to intracellular cAMP levels. The cAMP levels were highest in the RS, HLJDT, and HLJDT-M groups, while Cortex phellodendri and Rhizoma coptidis increased in level more than the DMSO treatment, but less than RS (Fig. 1B). In addition, exendin-4 also increased intracellular cAMP levels. Similarly, GLP-1 secretion in the NCI-H716 cells exhibited the same pattern as insulin secretion in the Min6 cells (Fig. 1C).
Fig. 1. Glucose-Stimulated Insulin Secretion and cAMP Levels in Min6 Cells Treated with Water Extracts of Herbs, and GLP-1 Secretion in NCI-H716 Cells.

After 8h of incubation in low-glucose DMEM with 20μg/ml of water extracts of Fructus schisandrae chinensis (FSC), Rhizoma Polygonati odorati (RPO), Cortex phellodendri (CP), Rhizoma cortisid (RC), Fructus gardenia (FG), Huang-lian-je-du-tang (HLJDT), and HLJDT-M were treated with the same extracts in low (2 mM) or high-glucose (20 mM) Krebs-Ringer buffer (KRB) for 30 min to measure insulin concentrations in the buffer in Min6 cells (A). In addition, intracellular cAMP levels were determined in Min6 cells at the end of the experiment with an ELISA kit (B). NCI-H716 cells were plated in 24-well plates coated with Matrigel and incubated for 7 h in RPMI media (pH 7.2) with DMSO or 20μg/ml of water extracts as used in the insulin secretion experiments. They were incubated for 1 h at 37°C and GLP-1 concentrations in the buffer were measured (C). The sample size (n) for each group was 6. The bars represent mean ± SD. There were significant differences among all the groups by one-way ANOVA at p < 0.05. **Values of the bars with different superscripts were significantly different by the Tukey test at p < 0.05. **Significantly different from the control at p < 0.01.

Table 1. Body Weight, Epididymal Fat, Energy Intake, Serum Glucose, and Insulin Levels after the 8-Week Experiment Period

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 14)</th>
<th>HLJDT (n = 15)</th>
<th>HLJDT-M (n = 15)</th>
<th>RS (n = 15)</th>
<th>FSC (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>318 ± 30*</td>
<td>274 ± 23*</td>
<td>305 ± 26b</td>
<td>288 ± 29*</td>
<td>313 ± 31*</td>
</tr>
<tr>
<td>Epididymal fat (g)</td>
<td>3.1 ± 0.5*</td>
<td>2.3 ± 0.4a</td>
<td>2.8 ± 0.5ab</td>
<td>2.6 ± 0.5b</td>
<td>3.0 ± 0.4**</td>
</tr>
<tr>
<td>Energy intake (Kcal/d)</td>
<td>96 ± 14</td>
<td>97 ± 14</td>
<td>103 ± 14</td>
<td>98 ± 14</td>
<td>107 ± 15</td>
</tr>
<tr>
<td>Serum glucose (mm)</td>
<td>7.5 ± 0.9a</td>
<td>6.0 ± 0.8b</td>
<td>5.5 ± 0.7b</td>
<td>6.9 ± 0.8*</td>
<td>5.9 ± 0.8*</td>
</tr>
<tr>
<td>Serum insulin (ng/ml)</td>
<td>0.53 ± 0.07b</td>
<td>0.68 ± 0.09a</td>
<td>0.64 ± 0.08*</td>
<td>0.61 ± 0.07a</td>
<td>0.55 ± 0.08**</td>
</tr>
<tr>
<td>HOMA_{IR}</td>
<td>5.4 ± 0.7a</td>
<td>5.5 ± 0.8*</td>
<td>4.8 ± 0.7b</td>
<td>5.7 ± 0.8*</td>
<td>4.4 ± 0.7bs</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

*Significant difference among the groups in the same row by one-way ANOVA at p < 0.05.
**Values in the same row with different superscripts were significantly different by the Tukey test at p < 0.05.

Body weight, epididymal fat pads, energy intake, and serum glucose and insulin levels

HLJDT reduced body weight and visceral fat mass more than the control, and its component, RS, also decreased them (Table 1), but HLJDT-M and FSC did not bring about significant reductions. Caloric intake calculated by multiplying food intake by energy density was not significantly different among the groups. HLJDT, HLJDT-M, and FSC lowered fasting glucose concentrations in Px rats in comparison to the control group, but RS did not reduce serum glucose levels. Overnight fasting serum insulin levels rose in the rats administered HLJDT, HLJDT-M, and RS. HLJDT-M and FSC decreased the homeostasis model assessment of insulin resistance (HOMA_{IR}) in the Px rats as compared to the control groups, which indicates that HLJDT-M...
and FSC reduced insulin resistance (Table 1). Thus HLJDT and RS appeared to increase insulin secretion and FSC tended to reduce insulin resistance while HLJDT-M appeared to improve both.

**HLJDT-M improved glucose tolerance**

Only HLJDT-M significantly reduced serum glucose levels at the peak, but only HLJDT-M decreased the levels at faster rates than the control (Fig. 2A). HLJDT did not reduce serum glucose levels as much as HLJDT-M did after the peak during OGTT, while HLJDT-M exhibited a faster reduction of serum glucose levels than any other group (Fig. 2A). The rats that consumed FSC showed similar serum glucose levels at the peak to the control rats, but their levels fell to the baseline on a steeper slope than the control, similarly to RS.

The areas under the curves of serum glucose during OGTT are shown in Fig. 2B. As characterized in the OGTT curve, after the oral glucose load, administration of HLJDT-M to the Px rats resulted in a significantly lower area under the curve of serum glucose, compared to the control group ($p < 0.05$). However, the area under the curve of serum insulin was higher in the rats administered HLJDT-M, HLJDT, and RS than for those treated with cellulose as a control. Furthermore, FSC did not increase the area in comparison with the control. Thus the changes in serum glucose levels during OGTT were reflected by endogenous insulin secretion and insulin resistance.

**HLJDT-M and HLJDT improved insulin secretion capacity**

To confirm β-cell function, hyperglycemic clamping was performed on rats administered HLJDT-M, HLJDT, FSC, or RS for 8 weeks. During hyperglycemic clamping, serum insulin levels peaked between 0 and 5 min, and then significantly declined at 10 min during the steady infusion of glucose. This is known as first-phase insulin secretion. An ascending second-phase of plasma insulin was observed at 60 to 120 min in all the rats, when glucose levels remained elevated and stable. Insulin secretion capacity was represented by serum insulin levels during the first- and second-phases. Serum glucose levels increased until 60 min and remained at 5.5 mm above the baseline, and were similar among all the groups (Fig. 3A). Only HLJDT-M caused serum insulin levels to rise at 2 and 5 min after bolus glucose infusion (Fig. 3B). This indicates that HLJDT-M potentiated first-phase insulin secretion more than the other treatments, and this was confirmed by the area under the curve of serum insulin at the first-phase during hyperglycemic clamping (Table 2). HLJDT, RS, and FSC also enhanced first-phase insulin secretion better than the control, but not as much as HLJDT-M (Table 2). However, serum insulin levels at 60 to 120 min were strengthened by HLJDT-M, HLJDT, and RS in comparison with the control, which was comparable to the area under the curve of serum insulin at the second-phase. This suggests that HLJDT-M promoted glucose-stimu-
Insulin Secretion Patterns under Hyperglycemic Clamping

Hyperglycemic clamping was performed on rats subjected to overnight fasting to determine insulin secretion after 8-week experimental periods. During the hyperglycemic clamp procedure to maintain blood glucose levels at 5.5 mm above fasting levels, serum glucose levels (A) and serum insulin levels (B) were measured in 90% pancreatectomized diabetic rats administered water extracts of 200 mg of Fructus schisandrae chinensis (FSC), Radix scutellariae (RS), Huang-lian-jie-du-tang (HLJDT), or HLJDT-M per kg of body weight for 8 weeks. The sample size (n) for each group was the same as in Table 1, and the results were expressed as mean ± SD. *There were significant differences among all the groups at each time point, 2, 5, 60, 90 and 120 min, by one-way ANOVA at p < 0.05. a,b Values of the points with different superscripts were significantly different by the Tukey test at p < 0.05.

Fig. 3. Insulin Secretion Patterns under Hyperglycemic Clamping.

Table 2. Insulin Secretion Capacity and Insulin Sensitivity in the Hyperglycemic State during Hyperglycemic Clamping

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 14)</th>
<th>HLJDT (n = 15)</th>
<th>HLJDT-M (n = 15)</th>
<th>RS (n = 15)</th>
<th>FSC (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum insulin at basal state (ng/ml/min)</td>
<td>0.51 ± 0.09^b</td>
<td>0.66 ± 0.1^a</td>
<td>0.67 ± 0.09^e</td>
<td>0.66 ± 0.09^a</td>
<td>0.56 ± 0.10^b</td>
</tr>
<tr>
<td>Serum insulin at first-phase (ng/ml/min)</td>
<td>2.4 ± 0.4^d</td>
<td>3.1 ± 0.4^a</td>
<td>3.7 ± 0.4^e</td>
<td>2.9 ± 0.4^d</td>
<td>2.9 ± 0.4^d</td>
</tr>
<tr>
<td>Serum insulin at second-phase (ng/ml)</td>
<td>2.6 ± 0.3^a</td>
<td>3.4 ± 0.5^a</td>
<td>3.5 ± 0.5^a</td>
<td>3.2 ± 0.4^a</td>
<td>2.4 ± 0.3^a</td>
</tr>
<tr>
<td>Glucose infusion rate (mg/kg bw/min)</td>
<td>4.4 ± 0.7^c</td>
<td>5.4 ± 0.8^b</td>
<td>6.6 ± 0.8^b</td>
<td>5.1 ± 0.8^b</td>
<td>5.0 ± 0.7^b</td>
</tr>
<tr>
<td>Insulin sensitivity (μmol glucose-min⁻¹·100 g⁻¹ per μmol insulin/l)</td>
<td>23.9 ± 3.8^b</td>
<td>22.4 ± 3.6^b</td>
<td>26.2 ± 4.3^a</td>
<td>22.5 ± 3.5^b</td>
<td>29.4 ± 4.2^a</td>
</tr>
</tbody>
</table>

Values are mean ± SD. First-phase insulin secretion was calculated as the areas under the curves of serum insulin per min during 0–10 min under hyperglycemic clamping, while the second-phase was during 60–120 min. Data were represented as the area under the curve divided by the time period. Glucose infusion rates were the average of glucose infusion rates during 60–120 min to maintain hyperglycemic states under hyperglycemic clamping. Insulin sensitivity under hyperglycemic state was calculated as the ratio of the glucose infusion rate to steady-state plasma insulin levels.

*Significant difference among the groups in the same row by one-way ANOVA at p < 0.05.

a,b Values in the same row with different superscripts were significantly different by the Tukey test at p < 0.05.

Glucose infusion rates under hyperglycemic clamping indicated insulin sensitivity in the hyperglycemic state, which was calculated as the ratio of glucose infusion rates to steady-state serum insulin levels. Glucose infusion rates to maintain serum glucose levels at 5.5 mm above baseline were found to be greater in the ascending order of control, FSC, RS, HLJDT, and HLJDT-M (Table 2). The rates were significantly lower in the control than the various treatments. Insulin sensitivity in the hyperglycemic state was higher in HLJDT-M and FSC than the control, but was not different in RS, HLJDT, and the control. Thus the results of hyperglycemic clamping revealed that HLJDT-M, HLJDT, RS, and FSC improved glucose homeostasis by independent pathways in hyperglycemic states. HLJDT-M enhanced it by potentiating glucose-stimulated insulin secretion capacity and insulin sensitivity, but HLJDT and RS improved homeostasis only by promoting insulin secretion capacity, while FSC improved it only by promoting insulin sensitivity (Table 2).
mRNA Levels of IRS2, PDX-1, and Glucokinase in the Islets.

At the end of the 8-week experimental period, islets were isolated from each group of Px rats fed water extracts of 200 mg of Fructus schisandrae chinensis (FSC), Radix scutellariae (RS), Huang-lian-jie-du-tang (HLJDT), or HLJDT-M per kg of body weight for 8 weeks and mRNA levels of IRS2, PDX-1, and glucokinase were measured by real-time PCR. The bars represent mean ± SD. The sample size (n) for each group was 5. There were significant differences among the groups by one-way ANOVA at p < 0.05. aValues of the bars with different superscripts were significantly different by the Tukey test at p < 0.05.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>β-cell area (%)</th>
<th>Individual β-cell size (μm²)</th>
<th>Absolute β-cell mass (mg)</th>
<th>BrdU⁺ cells (% BrdU⁺ cells of islets)</th>
<th>Apoptosis (% apoptotic bodies of islets)</th>
<th>Ratio of β/α cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.7 ± 0.8b</td>
<td>236.2 ± 29.3ᵃ</td>
<td>21.5 ± 3.3ᵇ</td>
<td>0.79 ± 0.10ᵇ</td>
<td>0.70 ± 0.08ᵇ</td>
<td>4.8 ± 0.7ᵇ</td>
</tr>
<tr>
<td>HLJDT</td>
<td>7.0 ± 0.9ᵃ</td>
<td>207.5 ± 26.5ᵇ</td>
<td>29.7 ± 4.5 aVar</td>
<td>0.94 ± 0.12ᵇ</td>
<td>0.68 ± 0.08ᵇ</td>
<td>5.1 ± 0.7ᵇ</td>
</tr>
<tr>
<td>HLJDT-M</td>
<td>7.7 ± 0.9ᵃ</td>
<td>182.5 ± 25.3ᵇ</td>
<td>28.0 ± 3.6ᵇ</td>
<td>1.09 ± 0.13ᵇ</td>
<td>0.60 ± 0.08ᵇ</td>
<td>5.9 ± 0.7ᵇ</td>
</tr>
<tr>
<td>RS</td>
<td>7.7 ± 0.9ᵃ</td>
<td>210.7 ± 25.6ᵇ</td>
<td>27.5 ± 3.4ᵇ</td>
<td>0.91 ± 0.11ᵇ</td>
<td>0.69 ± 0.08ᵇ</td>
<td>4.9 ± 0.7ᵇ</td>
</tr>
<tr>
<td>FSC</td>
<td>7.1 ± 0.7ᵇ</td>
<td>185.6 ± 26.7ᵇ</td>
<td>23.9 ± 3.5ᵇ</td>
<td>0.83 ± 0.10ᵇ</td>
<td>0.61 ± 0.07ᵇ</td>
<td>5.4 ± 0.8ᵇ</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

*Significantly different among the groups in the same row by one-way ANOVA at p < 0.05.

**Values in the same row with different superscripts were significantly different by the Tukey test at p < 0.05.

### Intracellular cAMP levels and IRS2, PDX-1, and glucokinase expression in the islets

The islets isolated from the rats administered HLJDT-M (2.6 ± 0.5 pmol/mg of protein), HLJDT (2.4 ± 0.4 pmol/mg of protein), and RS (2.3 ± 0.5 pmol/mg of protein) had increased intracellular cAMP levels in comparison to the control (0.8 ± 0.4 pmol/mg of protein) and FSC (0.9 ± 0.4 pmol/mg of protein). In parallel with cAMP levels, the mRNA levels of IRS2, PDX-1, and glucokinase were higher in islets isolated from the rats administered HLJDT-M, HLJDT, and RS than in the control (Fig. 4). FSC did not alter the mRNA levels of these genes in the islets as compared to control (Fig. 4).

**HLJDT-M, HLJDT, and RS increased β-cell mass by increasing β-cell proliferation**

The percentage of the β-cell area in the total pancreas area of a section was significantly higher in HLJDT-M, HLJDT, and RS fed Px rats as compared to control (Table 3), but pancreas weight was not significantly different among the various treatments (data not shown). Thus pancreatic β-cell mass, calculated by multiplying the β-cell area by the pancreas weight, was increased by HLJDT-M, HLJDT, and RS in the Px rats as compared to control (Table 3). β-cell mass was characterized by individual cell size and numbers, and the means by which β-cell mass was increased differed among the groups. HLJDT-M increased β-cell mass by elevating the numbers of β-cells without enlarging individual β-cell size, but HLJDT and RS increased the numbers of β-cells in comparison to control, while they decreased individual β-cell size as compared to control. This suggests that existing hypertrophy resulted from increased insulin resistance, as seen in the results for HOMAIR (Table 1). In contrast, HLJDT-M increased the number of β-cells (hyperplasia) by enhancing proliferation and reducing apoptosis in the Px rats, but it did not induce hypertrophy of β-cells (Table 3). FSC did not increase β-cell proliferation in comparison to control, but it reduced apoptosis. However, this reduction of apoptosis was not sufficient to increase β-cell mass in the FSC-treated rats (Table 3). While HLJDT-M lowered β-cell apoptosis in the Px rats more than in control, HLJDT and RS did not alter apoptosis in comparison with control. The ratio of β-cells to α-cells was higher in the HLJDT-M and FSC fed Px rats than in control, while the other treatments did not change the ratio (Table 3). Thus, HLJDT-M, HLJDT, and RS increased β-cell mass in independent ways.

### Discussion

Insulin secretion and insulin action are essential for glucose homeostasis, and defects in either process cause glucose intolerance that can progress to type-2 diabetes and other serious metabolic disorders. Recent studies of experimental animals have shown that a failure of insulin secretion causes the development of type-2 diabetes, which is associated with decreased β-cell expansion. Hence it is crucial to find herbs that improve glucose-stimulated insulin secretion by accompanying β-cell expansion by hyperplasia in order to prevent the prevalence and progression of diabetes. The present and other preliminary studies indicate that RS
and Cortex phellodendri enhance glucose-stimulated insulin secretion in Min6 cells by increasing intracellular cAMP levels. They also elevated GLP-1 secretion in NCI-H716 cells. Their effects are associated with berberine, a major component of Rhizoma coptidis. Berberine was found to increase insulin-stimulated glucose uptake and to reduce triglyceride storage in 3T3-L1 adipose tissues by activating AMPK. It also possessed a property that enhanced glucose-stimulated insulin secretion and proliferation in Min6 cells by a potentiated insulin/insulin-like growth factor-1 signaling cascade. However, high dosages of berberine cannot be used in treatment, since over 100 μm berberine has been found to show cytotoxicity in cancer and normal cells. Although the extract of Rhizoma coptidis was in our previous study not to be as effective as berberine, it was to be less toxic. In addition, RS, Rhizoma coptidis, Cortex phellodendri were to alleviate metabolic syndromes, including type-2 diabetes, hypercholesterolemia, and hypertension in our previous studies. Hence we tried to find a remedy that would include the components, RS, Cortex phellodendri, and Rhizoma coptidis. This remedy was HLJDT.

There are several pieces of evidence to support the hypothesis that the elevation of intracellular cAMP enhances insulin secretion and β-cell mass. Increased levels of cAMP are a known mechanism enhancing glucose-stimulated insulin secretion. Increased levels of cAMP activate protein kinase A (PKA), which can close the K<sub>ATP</sub> channels, and they also activate Ca<sup>2+</sup> channels and the exocytosis of insulin, which contains granules. Exendin-4 also increases intracellular cAMP levels by activating GLP-1 receptors, which induce glucose-stimulated insulin secretion in Min6 cells. Yue et al. reported that baicalin, a major flavonoid derived from RS and Cortex phellodendri, was also found to potentiate Ca<sup>2+</sup>-mediated Cl<sup>−</sup> secretion through a signaling pathway involving cAMP and protein kinase A in the apical membrane of intestinal epithelial cells. Thus RS, HLJDT, and HLJDT-M acted directly on β-cells to potentiate glucose-stimulated insulin secretion and to sustain β-cell expansion, but they did not increase glucose-stimulated insulin secretion as much as exendin-4. This might be related to the increase of intracellular cAMP levels.

The mechanism involved in potentiating GLP-1 secretion might be the same mechanism that enhances glucose-stimulated insulin secretion from β-cells. GLP-1 is a gastrointestinal hormone secreted in response to meal ingestion by enteroendocrine L-cells located predominantly in the lower small intestine and the large intestine of humans and animals. In vivo, GLP-1 circulates in the blood stream and acts on β-cells, the brain, and other tissues. It acts on β-cells to increase intracellular cAMP by activating G-protein coupled GLP-1 receptors and it works as an insulinotropic agent. Similarly to insulin secretion, RS, HLJDT, and HLJDT-M increased GLP-1 secretion in NCI-H716 cells in the present study. Hence we hypothesize that RS, HLJDT, and HLJDT-M were able to improve insulin secretion and β-cell expansion indirectly through elevating GLP-1 secretion. However, GLP-1 cannot be used as a therapeutic drug, since it is quickly inactivated by dipeptidyl peptidase (DPP)-IV upon secretion. Several GLP-1 receptor agonists, including exendin-4 and DPP-IV inhibitors, have been to relieve diabetic symptoms. The activity of exendin-4 continues for 12 h and has biological effects similar to that of GLP-1. RS, Cortex phellodendri, HLJDT, and HLJDT-M increase intracellular cAMP in β-cells directly, enhancing glucose-stimulated insulin secretion in experimental animals and humans. In addition, they might indirectly potentiate insulin secretion by increasing GLP-1 secretion from L-cells, but this indirect action might not be substantial, due to GLP-1 degradation upon secretion.

Similarly to the in vitro experiments, RS, HLJDT, and HLJDT-M potentiated insulin secretion in the Px diabetic rats. Our previous study showed that exendin-4 treatment improved first- and second-phase insulin secretion under hyperglycemic clamping in the Px rats. Exendin-4 enhanced first-phase insulin secretion better than second-phase under hyperglycemic clamping, but exendin-4 prevented an abrupt decrease in insulin secretion at 120 min. HLJDT-M improved insulin secretion in a pattern similar to exendin-4. As mentioned above, insulin secretion is coupled directly to the activation of a cAMP-dependent mechanism in HLJDT-M as well as exendin-4 treatment. In addition to a cAMP dependent pathway, improvement of first-phase insulin secretion was related to a reduction in peripheral insulin resistance and induction of glucokinase, promoting glucose sensing. Glucokinase serves as a glucose sensor in pancreatic β-cells and regulates glucose-stimulated insulin release. Increased insulin resistance due to a high-fat diet, obesity, and other conditions is known to reduce both GLUT-2 and glucokinase expression, thereby impairing glucose-stimulated insulin secretion. Furthermore, a high-fat diet and other conditions induce insulin resistance, oxidative stress, and apoptosis, and reduce glucose-stimulated insulin secretion. Exendin-4 has been found to increase glucokinase expression in the islets. Similarly to exendin-4, RS, HLJDT, and HLJDT-M induced mRNA expression of glucokinase in the present study. This induction was positively associated with insulin secretion under hyperglycemic clamping.

In addition to potentiating insulin secretion, increased intracellular cAMP activates PKA → pCREB in β-cells. Morris F. White has put forward evidence indicating that CRE site binding to pCREB exists in the upstream of the IRS2 coding region in genes of humans and mice. The activation of CREB, an important cell survival factor, increased IRS2 expression. Sarkar et al. found that impairment of CREB-mediated transcription resulted in loss of human islets by apoptosis, leading to diabetes. IRS2 induction potentiated the IRS2 → P13 kinase → Akt cascade in islets, which induced PDX-1 expression, promoting the growth and survival of β-cells and insulin synthesis in rodents and humans. PDX-1 is required for the development of the pancreas in mice and humans, but it also promotes β-cell function in adults. Genetic disruption of the IRS2 or the PDX-1 gene in β-cells promotes apoptosis, which can lead to diabetes. Thus the increment of cAMP in β-cells raises β-cell proliferation increasing β-cell mass and improves glucose-stimulated insulin secretion. The present study also indicates that RS, HLJDT, and HLJDT-M increased intracellular cAMP inducing
IRS2 mRNA levels. This was related to elevated \( \beta \)-cell mass. Increased \( \beta \)-cell mass due to hyperplasia is necessary to sustain glucose-stimulated insulin secretion, leading to improvement of anti-diabetic symptoms.

In summary, RS, HLJDT, and HLJDT-M enhanced glucose-stimulated insulin secretion and increased \( \beta \)-cell mass in \( \text{Px} \) diabetic rats. However, the mechanism by which they did so was not the same: HLJDT-M improved glucose tolerance whereas RS and HLJDT did not enhance it. This difference affected insulin secretion and islet morphometry. Only HLJDT-M increased both first- and second-phase insulin secretion. HLJDT and RS did not improve first-phase insulin secretion as much as HLJDT-M but they did elevate second-phase insulin secretion under hyperglycemic clamping. HLJDT-M elevated \( \beta \)-cell hyperplasia the most among the groups, but it decreased individual \( \beta \)-cell size. RS and HLJDT reduced the hypertrophy of \( \beta \)-cells and increased \( \beta \)-cell hyperplasia, but not as much as HLJDT-M. The increase in hyperplasia was positively related to elevated IRS2 and PDX-1 expression in the islets. In conclusion, RS and HLJDT are not adequate anti-diabetic prescriptions, since they did not properly improve glucose tolerance, even though they enhanced insulino-motrophic action. HLJDT-M is a better anti-diabetic prescription than HLJDT or RS. Without enhancing insulin sensitivity, herbs that potentiate insulino-motrophic action are limited in their ability to treat diabetic symptoms.

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**References**