Original Article

Standardized chungkookjang, short-term fermented soybeans with *Bacillus lichemiformis*, improves glucose homeostasis as much as traditionally made chungkookjang in diabetic rats

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As the traditional homemade chungkookjang is replaced by standardized chungkookjang fermented by inoculating *Bacillus* spp., it is desirable to maintain the anti-diabetic efficacy of the most potent traditional varieties. Preliminary *in vitro* research suggested that anti-diabetic efficacy can be achieved by using *B. lichemiformis* as a starter and fermenting for 48 h. Experimental type 2 diabetic male rats induced by partial pancreatectomy and high fat diets were administered either control diet, 10% cooked soybeans, 10% traditional chungkookjang with potent anti-diabetic efficacy, or standardized chungkookjang fermented with *B. lichemiformis* for 48 h. Rats were fed their respective diets for 8 weeks after surgery. Cooked soybeans as well as both chungkookjangs partially restored fasting serum glucose concentrations, but only the chungkoojangs increased fasting insulin levels. That trend was also seen in the glucose-stimulated insulin secretion during hyperglycemic clamp and was explained by the greater β-cell mass and BrdU incorporation indicating increased proliferation of β-cells. The euglycemic hyperinsulinemic clamp indicated that all soy products improved insulin sensitivity. Phosphorylation of Akt and AMPK in the liver increased in an ascending order of the control, cooked soybeans, traditional chungkookjang and standardized chungkookjang while PEPCK expression was lowered in a descending order of the control, cooked soybeans, traditional chungkookjang and standardized chungkookjang. These results indicate that standardized chungkookjang is most effective for improving hepatic insulin signaling. In conclusion, chungkookjang fermented with *B. lichemiformis* retains the anti-diabetic properties of the most efficacious traditional chungkookjang and it may be even more effective for improving insulin function than traditionally prepared chungkookjang.

Key Words: short-term fermented soybeans, insulin sensitivity, insulin secretion, β-cell proliferation, hepatic glucose output

S oybeans (*Glycine max* MERILL) are an important plant protein source, complementing protein from grains to make complete proteins. Since Asians consume grains as staple components of their diets, soybeans are necessary to be consumed. Soybeans contain various nutritious and functional components such as isoflavonoids besides soy protein which are helpful in protecting against metabolic diseases such as obesity and type 2 diabetes.[1] Type 2 diabetes develops when insulin secretion cannot compensate for insulin resistance.[2] Anti-diabetic medications and functional foods need to improve insulin sensitivity and/or β-cell function and mass. Soybeans are reported to attenuate insulin resistance but it remains controversial whether they potentiate insulin secretion.

Fermenting soybean to make products such as chungkookjang, deonjang, soy sauce and kochujang potentiates the functionality of soybeans to protect against metabolic disease. Natto, a Japanese food similar to chungkookjang, and kochujang have been reported to have better anti-diabetic effects than unfermented soybeans in diabetic animals and humans.[3,4] Most fermented soybean products except chungkookjang contain high concentrations of salt as a preservative. Chungkookjang is a good fermented soybean product for use as a functional food. However, traditionally made chungkookjang lacks consistent efficacy as a functional food since its functionality varies according conditions during its preparation, such as regional differences in ambient microorganisms. Our previous studies have revealed that traditionally made chungkookjang from Sunchang, Korea improves insulinotropic action and hepatic insulin sensitivity in diabetic rats.[5,6] However, some traditionally made chungkookjang from other areas might have less anti-diabetic activity. Recently, we found that chungkookjang fermented with *Bacillus lichemiformis* (*B. lichemiformis*) for 48 h has potent anti-diabetic activity in cell-based studies, equivalent to traditionally made chungkookjang in Sunchang (Yang *et al.*, 2012, unpublished data). However, chungkookjang fermented with *B. subtilis* and *B. amylofenices* had less anti-diabetic activity. Thus, *B. licheniformis* may be one of the major microorganisms responsible for anti-diabetic actions of fermented soybeans. These differences may be related to the changes in isoflavonoids and peptides in different chungkookjangs. Individual isoflavonoid and peptide patterns of chungkookjang fermented in the traditional manner are similar to those of chungkookjang fermented with *B. lichemiformis* (Yang *et al.*, 2012, unpublished data). We hypothesized that the 8-week consumption of chungkookjang fermented traditionally or by the standardized method would similarly improve glucose homeostasis in diabetic rats. We therefore investigated the insulin sensitizing and insulinotropic actions of chungkookjang made in both the traditional and standardized

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manners in diabetic animals. The type 2 diabetes rat model was generated by the combination of a 90% pancreatectomy (Px) which results in about a 50% decrease in insulin secretory capacity after partial regeneration of the pancreas and by a high fat diet which induces insulin resistance.[7,8] Since the rats release sufficient insulin to avoid ketosis, they are a type 2 (not type 1) diabetic model. They are non-obese and have similar characteristics to Asian type 2 diabetic patients that are typically non-obese and have insulin insufficiency.[9] To the best of our knowledge, this is the first report on the anti-diabetic activity of chungkookjang, short-term fermented soybeans with *B. lichemiformis* in a type 2 diabetic animal model.

Materials and Methods

Preparation of traditional and standardized chungkookjang. TFC was prepared using a traditional processing method known to produce potent anti-diabetic efficacy at Institute of Sunchang Fermented Soybean Research Institutes (Sunchang, Korea).[5,6] Soybeans were sorted, washed and soaked in water for 12 h at 15°C and boiled for 4 h at 100°C. The cooked soybeans were cooled to 40°C and fermented with rice straw at 42°C for 48 h for traditional chungkookjang. Rice straw contained the environmental Bacilli to ferment soybeans in a traditional manner.

B. licheniformis SCD 111067P was obtained from Institute of Sunchang Fermented Soybean Research Institutes (Sunchang, Korea). *B. lichemiformis* was cultivated in Luria-Bertani broth at 37°C with shaking (128 rpm, Jeio Tech., Daejeon, Korea) to expand the number of *Bacillus* spp. and they were used to inoculate soybeans immediately after the culture. Standardized chungkookjang was prepared using modern methods. Briefly, soaked soybeans was sterilized at 121°C for 1 h, cooled to 40°C and inoculated with 1% (v/w) *B. licheniformis* KCCK 11054P at concentrations of 10^7–10^8 CFU·ml^{-1}. *B. lichemiformis* was selected since it showed anti-diabetic activity in our previous *in vitro* study (Yang *et al.*, 2012, unpublished data). Each of the inoculated soybean preparations was fermented at 42°C for 48 h and freeze-dried.

Animals and diets. Male Sprague Dawley rats, weighing 211 ± 14 g, were housed individually in stainless steel cages in a controlled environment (23°C and a 12 h light and dark cycle). All surgical and experimental procedures were performed according to the guidelines and with the approval of the Animal Care and Use Review Committee at Hoseo University, Korea. The rats had a 90% pancreatectomy (Px) using the Hosokawa technique or received a sham pancreatectomy surgery (Sham).[7] Px rats included in the experiments showed characteristics of

type 2 diabetes, while the Sham rats did not.

Px rats were randomly assigned to four different groups (control, CSB, TFC and SFC) of 20 animals each according to dietary protein source. All rats freely consumed water and corresponding diets for 8 weeks. Px diabetic rats as a control group and sham-operated non-diabetic rats as a normal-control were fed a casein diet. The experimental diets were semi-purified, modified AIN-93 formulations.[10] The diets in the CSB, TFC and SFC groups contained 10% lyophilized cooked soybeans, traditionally fermented chungkookjang or standardized chungkookjang, respectively, as a main protein source. The compositions of the diets were based on previous studies: since the effective dietary iso-flavoind contents have been shown about 0.01–0.05% of diets, diets including 10% lysophilized CSB and TFC/SFC contain about 0.03% total isoflavonoids.[11] Our unpublished study demonstrated that 5% of traditionally made chungkookjang was less effective than 10% in regulating glucose homeostasis (Yang *et al.*, 2012, unpublished data). Since chungkookjang contained a mixture of nutrients, their compositions were analyzed. Cooked soybeans contained 39.7, 18.4, 31.8 and 5.3% of protein, lipid, carbohydrates and dietary fiber, respectively while chungkookjang was composed of 38.8, 19.6, 30.7 and 5.8%, respectively. According to the results of the macronutrient analysis (carbohydrates, protein and lipids) of soybeans and chungkookjang, the composition was tailored to exhibit equal proportions in all diets by adding soybean oil and cellulose. Any insufficiency in the quantity of protein in each diet was corrected by adding casein, the protein source for the control group. All diets consisted of approximately 40 energy percent (En%) carbohydrates, 20 En% protein and 40 En% fats (Table 1). The differences among these diets were essentially the degree of hydrolysis of protein and the presence of isoflavones, mainly as glycosides or aglycones, as determined by the previous study (Yang *et al.*, 2012, unpublished data).

Oral glucose tolerance test (OGTT). An oral glucose tolerance test was performed in the sixth-week in overnight-fasted animals by orally administering 2 g glucose/kg 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 radioimmunoassay kit (Linco Research, Billerica, MA), respectively. The averages of the total areas under the curves for serum glucose and insulin levels were calculated by the trapezoidal rule. Since 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. Overnight-fasted serum leptin and non-esterified fatty acid (NEFA) levels were measured by radioimmunoassay kit

Table 1. Composition of experimental diets

	Casein diet	Cooked soybeans (CSB) diet	Chungkookjang (TFC/SFC) diet
Casein	200	165	167
Methionine	3	3	3
Corn starch	310	280	286
Sucrose	200	200	200
Cellulose	34	13	12
Corn oil	50	40	38
Shortening	150	150	150
Mineral	35	31	30
Vitamin	10	10	10
Choline	2	2	2
Powder*	0	100	100
Total isoflavonoids (%)	—	0.036	0.026
Isoflavonoid aglycones (%)	—	0.001	0.014

*Assigned dried powder of cooked soybeans or chungkookjang for each group.

(Linco Research) and NEFA C enzymatic kit (Waco Diagnostics, Richmond, VA), respectively.

Euglycemic hyperinsulinemic clamp. After catheterisation of the right carotid artery and left jugular vein in the 7th week, a euglycemic hyperinsulinemic clamp was performed on fasted conscious rats ($n = 10$) to determine insulin resistance as previously described.[8,12] [3-^{3}H] glucose (Perkin Elmer, Wellesley, MA) was continuously infused during a four-hour period at the rate of 0.05 µCi/min. Basal hepatic glucose output was measured in blood collected at 100 and 120 min after initiation of the [3-^{3}H] glucose infusion. A primed continuous infusion of human regular insulin (Humulin; Eli Lilly, Indianapolis, IN) was then initiated at a rate of 20 pmol 7 kg^{-1} 7 min^{-1} to raise plasma insulin concentration to approximately 1100 pM after 210–240 min. Blood samples from arteries were collected at 10-min intervals for glucose evaluation, and 25% glucose was infused as needed to clamp glucose levels at approximately 6 mM. Disintegrations per min (dpm) of plasma [3-^{3}H]-glucose with and without drying were measured; plasma concentration of ^{3}H$_2$O was determined by the difference between ^{3}H counts with and without drying. Rates of whole body glucose uptake and basal glucose turnover were determined according to the ratio of the [3-^{3}H] glucose infusion rate to the specific activity of plasma glucose (dpm/mmol) during the final 30 min. Hepatic glucose production at the hyperinsulinemic clamped state was determined by subtracting the glucose infusion rate from the whole body glucose uptake. After clamp, the rats were immediately anesthetised with a mixture of ketamine and xylazine and were killed by decapitation. Tissues were rapidly collected, weighed, frozen in liquid nitrogen, and stored at −70°C for further experiments.

Hyperglycemic clamp. After seven weeks of treatment, catheters were surgically implanted into the right carotid artery and left jugular vein of ten conscious and overnight fasted rats from each group after anesthetization with ketamine and xylazine (100 mg and 10 mg/kg body weight, respectively). After 5–6 days of implantation, a hyperglycemic clamp was performed in free-moving and overnight fasted rats to determine insulin secretory capacity as described in previous studies.[8,13] During the clamp, glucose was infused to maintain serum glucose levels of 5.5 mM above the baseline and serum insulin levels were measured at 0, 2, 5, 10, 60, 90 and 120 min. After the clamp, rats were freely provided with foods and water for 2 days, and on the next day they were deprived of food for 16 h. The rats were anesthetized with a mixture of ketamine and xylazine, and human regular insulin (5 U/kg body weight) was injected through the inferior vena cava of the rats. Ten min later, they were killed by decapitation and tissues were rapidly collected, frozen in liquid nitrogen, and stored at −70°C for further determinations. In order to determine the liver glycogen content, lysates were centrifuged at 3000 rpm for 10 min and the supernatants deproteinized with 1.5 N perchloric acid. The glycogen content was calculated from glucose from glycogen hydrolyzed by α-amyloglucosidase in an acid buffer.[14] Triglyceride was extracted with chloroform-methanol (2:1, vol/vol) from the liver and resuspended in pure chloroform.[15] After evaporating the chloroform, the residue was suspended in PBS with 0.1% triton X-100 and sonicated and boiled for 5 min. The triglyceride contents of the suspension were determined using a Trinder kit (Young Dong Pharm., Seoul, Korea).

Immunoblot analysis. The livers taken from four rats after hyperglycemic clamp were used for an immunoblotting assay.[5,8] The frozen livers from each rat were lysed with 20 mM Tris buffer (pH 7.4) containing 2 mM EGTA, 137 mM NaCl, 1% NP40, 10% glycerol, and 12 mM α-glycerol phosphate and protease inhibitors. After measuring protein contents in lysates (Biorad kit, Hercules, CA), equal amounts of protein in the lysates (30–40 µg) were resolved by SDS-PAGE and immunoblotted with phospho-Akt^{ser478}, Akt, phospho-AMPK^{thr172}, AMPK (Cell Signaling Technology, Beverly, MA), and phosphoenolpyruvate carboxykinase

(PEPCK), generously provided by Dr. Granner of Vanderbilt University. The primary antibody was diluted by 1000X and secondary antibody was diluted by 5000X. The intensity of protein expression was determined using Imagequant TL (Amersham Biosciences, Piscataway, NJ).

Immunohistochemistry and islet morphometry. At the end of the 8-week experimental period, nine to ten rats from each group were injected with BrdU (100 µg/kg body weight). Six hours post-injection, rats were anesthetized with intraperitoneal injections of a mixture of ketamine and xylazine, and the pancreas was immediately dissected. The pancreas was fixed with 4% paraformaldehyde and paraffin-embedded, as described in previous studies.[6,8] Two serial 5-µm paraffin-embedded tissue sections were selected out of the 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. 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 10x with a Zeiss Axiovert microscope (Carl Zeiss Microimaging, Thornwood, NY). The results of β-cell quantification were expressed as the percentage of the total surveyed area containing insulin-positive cells, measured by IP Lab Spectrum software (Scanalytics Inc., Fairfax, VA). Pancreatic β-cell mass was calculated by multiplying the percentage of insulin-positive area by the weight of the corresponding pancreatic portion.[7,12] The individual β-cell size was determined as the insulin-positive area divided by the number of nuclei counted in the corresponding insulin-positive structures in randomly immunofluoresence-stained sections.[16] Enlarged individual β-cell size indicates the induction of β-cell hypertrophy.[7] BrdU incorporation in β-cells was determined by staining rehydrated paraffin sections with anti-insulin and anti-BrdU antibodies and β-cell proliferation was calculated as the total BrdU$^+$ nuclei in β-cell nuclei per pancreas section.[6,8] Apoptosis of β-cells was measured using a TUNEL kit (Roche Molecular Biochemicals, Indianapolis, IN) and counterstained with hematoxylin and eosin to visualize islets and apoptotic β-cells was measured by the total number of apoptotic bodies in β-cell nuclei per pancreas section.[6,8]

Statistical analysis. Statistical analysis was performed using SAS software and all results expressed as mean ± SD. The biological and metabolic effects of casein (control), CSB, TFC and SFC were compared by one-way ANOVA. Significant differences in the main effects among the groups were identified by Tukey's test at $p<0.05$. The differences between the Px diabetic rats (control) and Sham non-diabetic rats (normal-control) were determined by two-sample t test.

Results

Body weight, caloric intakes, and overnight-fasting glucose, insulin and leptin levels. Body weight and epididymal fat pads were lower in Px rats than Sham rats and this was not related to caloric intake. Px rats consumed more calories than Sham rats (Table 2). Serum leptin levels were lowered in Px rats than Sham rats and this was probably due to less insulin secretion and less fat mass. Overnight-fasted serum glucose levels of Px were higher than Sham rats (Table 2). In addition, serum insulin levels of Px rats were lower than those of Sham rats, indicating that Px rats did not show hyperinsulinemia but serum insulin levels were sufficient to avoid ketosis. Px rats, therefore, exhibited non-obese type 2 diabetes. CSB had quite different effects on energy metabolism from TFC and SFC. CSB lowered body weight and epididymal fat pads in comparison to the control but TFC and SFC did not (Table 2). However, energy intakes were lower in CSB, TFC and SFC. Serum leptin levels were lower in CSB than the control but TFC and SFC were not different from control.

Table 2. Metabolic changes

	Control (n = 20)	CSB (n = 20)	TFC (n = 20)	SFC (n = 20)	Normal-control (n = 20)
Body weight (g)	348 ± 32[a]	299 ± 24[b]	323 ± 31[a,b]	316 ± 32[a,b]	435 ± 41[†]
Epidydimal fat pads (g)	3.9 ± 0.6[a]	2.1 ± 0.5[c]	3.2 ± 0.6[b]	3.1 ± 0.5[b]	4.6 ± 0.7[†]
Caloric intakes (kcal/day)	123 ± 18.5[a]	93.5 ± 11.3[b]	105 ± 14.3[b]	106 ± 14.2[b]	94.2 ± 12.1[†]
Overnight fasted leptin levels (ng/mL)	5.3 ± 0.7[a]	3.6 ± 0.6[b]	4.7 ± 0.7[a]	4.8 ± 0.7[a]	6.7 ± 0.9[†]
Overnight fasted serum glucose (mmol/L)	8.4 ± 1.2[a]	7.1 ± 1.0[b]	6.7 ± 0.9[b]	6.8 ± 0.9[b]	4.4 ± 0.6[†]
Overnight fasted serum insulin (ng/mL)	0.78 ± 0.12[b]	0.73 ± 0.14[b]	1.03 ± 0.21[a]	1.01 ± 0.19[a]	1.42 ± 0.21[†]

Values are mean ± SD. Diabetic rats in the control, CSB, TCF and SFC groups were provided with 40 En% fat diets added with casein, 10% cooked soybean, chungkookjang made with traditional manner and chungkookjang made with standard manner, respectively. [a,b]Values in the same row with different superscripts were significantly different in Tukey's test at $p<0.05$. [†]The normal-control group of non-diabetic rats were significantly different from the control group of Px rats at $p<0.05$ by two sample t test.

Overnight-fasted serum glucose levels were lowered in the CSB, TFC and SFC groups than the control. Serum insulin levels increased in TFC and SFC, but not in CSB, in comparison to the control group (Table 2). The improvement in glucose metabolism with TFC and SFC in Px rats was not seen in Sham rats. SFC was comparable to TFC in improvement of energy and glucose metabolism while CSB exhibited differences in energy and glucose metabolism in comparison to the TFC and SFC.

Changes of glucose tolerance. After the oral glucose loading, serum glucose levels increased up to 50 min and then slowly decreased in all rats (Fig. 1A). Serum glucose peaks were much higher and decreased more slowly in Px rats than Sham rats during OGTT. The area under the curve of first and second phase insulin secretion during OGTT was much greater in Sham rats than Px rats (Fig. 1B). This indicated that Px rats had glucose intolerance that was partly associated with impaired insulin secretion. TFC and SFC ameliorated glucose intolerance compared to control while the improvement of glucose tolerance by CSB was not significantly different from the control. SFC decreased serum glucose levels at peak in comparison to CSB and TFC but it was not significantly different (Fig. 1A). From the peak, the levels were decreased with similar patterns among CSB, TFC and SFC and the decrease was significantly different from the control. The changes of serum glucose levels during OGTT were confirmed by the area under the curve of glucose. The differences might be related to serum insulin levels. Serum insulin levels of the first phase of OGTT, representing an acute response to increased serum glucose levels, were higher in TFC and SFC than the control and CSB (Fig. 1B). Serum insulin levels of the second phase were not significantly different among the groups.

Insulin secretion capacity measured by hyperglycemic clamp. Fig. 2 shows the patterns of serum insulin levels during hyperglycemic clamp. Serum insulin levels were divided into two phases, first and second phase, by raising serum insulin levels. Serum insulin levels at the first phase and second phase were much higher by about 1.8–1.9 folds in Sham rats than Px rats (Table 3). In addition, insulin sensitivity at hyperglycemic state was lower in Px rats compared to Sham rats. This indicated that Px rats had insulin insufficiency and insulin insensitivity at hyperglycemic states. Serum insulin levels of Sham rats were not shown in Fig. 2 since the difference in the values of Px and Sham rats was too large to show in the same figure, but they are shown in Table 2. Serum insulin levels at the first phase increased in the ascending order of the control, CSB, TFC and SFC, while those at the second phase were significantly higher in TFC than the control (Table 3). The levels at second phase were greater in SFC than the control ($p<0.07$). The secretion pattern revealed that Px rats had a significant decrease at 120 min, which indicated that islets were too exhausted to secrete insulin after 90 min of insulin secretion (Fig. 2). The exhaustion was not present with CSB, TFC and SFC treatment. Thus, TFC and SFC partly restored insulin secretion and insulin sensitivity at hyperglycemic states in Px, rats but not to

the level of Sham rats.

Islet morphometry. Since the exhaustion of β-cells might be related to changes in β-cell mass, β-cell mass was determined. The β-cell area was greater in Px rats than Sham rats, causing them to release more insulin from a smaller size of pancreas (Table 4). However, β-cell mass was greater in Sham rats than Px rats due to the bigger pancreas by about 2 folds (data not shown) and the ratio of β:α cells was greater in Sham rats than Px rats (Table 4). TFC and SFC increased β-cell mass by increasing β-cell proliferation and decreasing β-cell apoptosis in comparison to the control (Table 4). However, CSB did not increase β-cell mass, due to no impact on β-cell proliferation. CSB, TFC and SFC lowered individual β-cell size (hypertrophy) more than the control, indicating that they would exhaust β-cells less than the control (Table 4). In addition, the ratio of β:α cells was not deteriorated in CSB, TFC and SFC in comparison to the control.

Insulin sensitivity measured by hyperinsulinemic euglycemic clamp. Px diabetic rats exhibited greater insulin resistance than Sham non-diabetic rats by decreasing glucose disposal rates and whole-body glucose uptake, and increasing hepatic glucose output at baseline and in hyperinsulinemic states (Fig. 3 A and B). Thus, Px rats fed high fat diets had type 2 diabetic characteristics of insufficient insulin secretory capacity to compensate for exacerbated insulin resistance. The increase in insulin resistance, as represented by lowered glucose infusion rats to normalize serum glucose levels at a hyperinsulinemic state, in Px rats was blocked with CSB, TFC and SFC in comparison to the control (Fig. 3A). Whole-body glucose uptake did not increase in any groups, but CSB, TFC and SFC resulted in an incremental increase in glucose disposal rates in comparison to the control (Fig. 3A). Hepatic insulin sensitivity was determined by hepatic glucose production at baseline and in hyperinsulinemic clamp states. At baseline, hepatic glucose output was consistent with fasting serum glucose levels but was significantly lower only in TFC and SFC. Hepatic glucose productions in hyperinsulinemic states were lower in TFC- and SFC-treated rats compared to control rats (Fig. 3B). These results suggested that CSB, TFC and SFC improved hepatic insulin sensitivity compared to the control.

Hepatic insulin signaling. After the hyperglycemic clamp study 4 rats from each group were selected for studying hepatic insulin signaling while still in a fed state. Hepatic Akt was much more phosphorylated in the SFC group than all other groups, although it was significantly greater in CSB and TFC than control (Fig. 4). A similar trend was seen for AMPK with phosphorylation being much higher for all soy food groups than for control. Among the soy food groups, phosphorylation was significantly greater for SFC than CSB but TFC was an intermediate value and was not significantly different from either CSB or SFC (Fig. 4). PEPCK expression was much higher in the control group than any of the other groups; SFC had significantly lower PEPCK expression than any of the other groups.
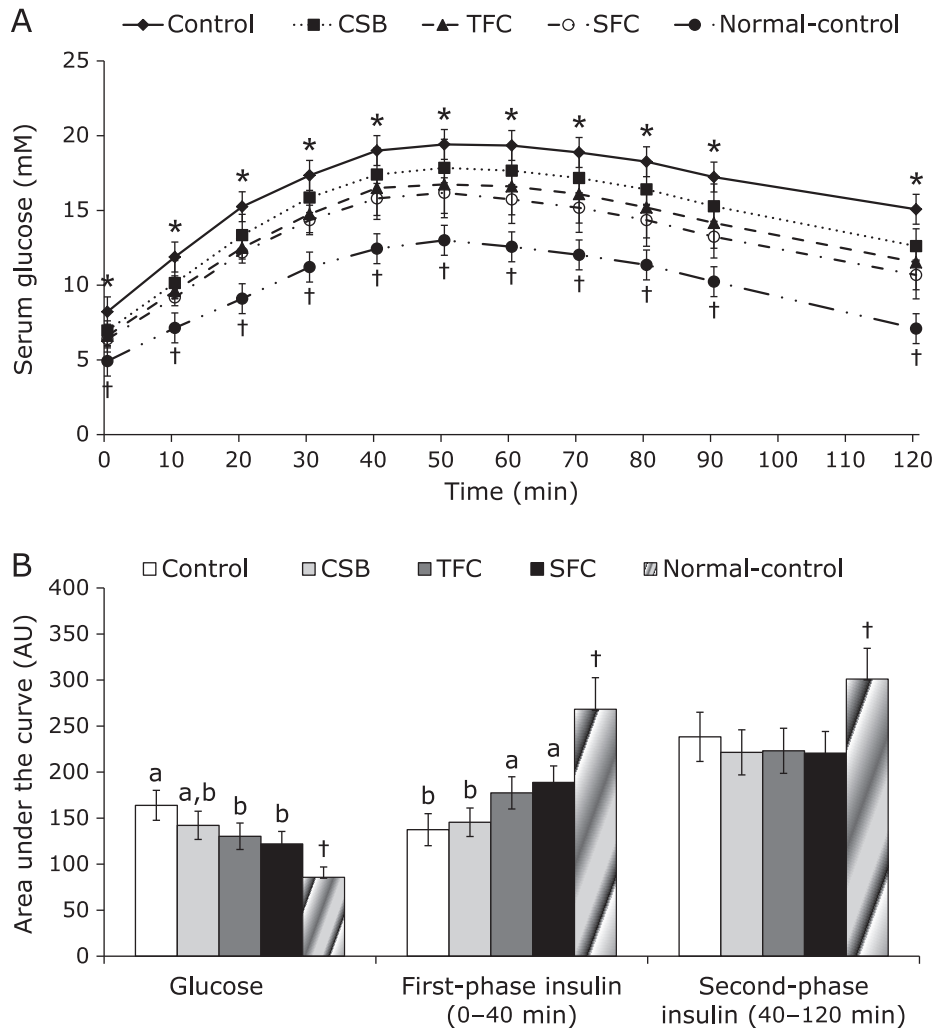
Fig. 1. The changes in serum glucose levels and the area under the curve for serum glucose and insulin during oral glucose tolerance testing. Oral glucose tolerance tests were performed on Px rats fed diets containing 10% cooked soybeans (CSB), chungkookjang made in the traditional (TFC) or standardized (SFC) manner, or casein (control), for 8 weeks following oral loading with 2 g glucose per kg body weight. After blood samples were taken at the time points indicated, serum glucose (A) and insulin levels were measured, and the area under the curve for glucose and insulin was calculated (B). The sample size in each group was the same as in Table 2. *Significantly different among groups of Px rats at $p<0.05$. [a,b]Values in the same row with different superscripts were significantly different in Tukey's test at $p<0.05$. [†]Significantly different from Px control at $p<0.05$.

Discussion

It is difficult to control the functional quality of Korean traditionally fermented foods prepared at home since the fermentation conditions can be different due to differences seasonal and regional conditions and ambient bacteria. In addition, traditionally fermented foods are rapidly being replaced by those made with a standard method for quality control and mass production. As with similar trends worldwide, these developments have the potential to either improve or diminish the nutritional value of the Korean diet. This study demonstrated that both a traditional chungkookjang, selected for it potent anti-diabetic properties, and a standardized chungkookjang, inoculated with *B. lichemiformis* as a fermentation starter, are equally effective for improving glucose tolerance in a type 2 diabetes rat model. A few other studies have indicated anti-diabetic effects of chungkookjang in mice[17] and rats[5,6,18] and there is some evidence that it may help decrease visceral fat and improve cardiovascular health in humans.[19] However, this study expanded the understanding of how chungkookjang can help prevent type 2 diabetes and

demonstrated that commercially manufactured chukookjang can provide health benefits that rival the very best of traditionally made chungkookjangs and may be superior in some respects.

Most studies have reported that *B. subtillus* is the major microorganism in traditionally made chungkookjang, but other microorganisms such as *B. licheniformis* and *B. amyloliquefaciens* are also present.[20,21] Previous studies have revealed that chungkookjang fermented with *B. licheniformis* SCD 111067P and *B. amyloliquefaciens* CH-86-1 contained more γ-polyglutamic acid (PGA) than chungkookjang made with *B. subtillus*. PGA is a commonly used indicator of chungkookjang quality.[20,22] In addition, chungkookjang fermented with *B. licheniformis* was more effective for increasing insulin-stimulated glucose uptake in 3T3-L1 adipocytes and glucose-stimulated insulin secretion in inusulinoma cells than that made with *B. subtillus* in our previous study (Yang *et al.*, 2012, unpublished data). This was associated with similar patterns of isoflavonoid aglycones and small peptides in traditionally made chungkookjang and standardized chungkookjang fermented with *B. lichemiformis*. Total isoflavonoid aglycones were greater in all kinds of chungkookjang by 4–9 folds in
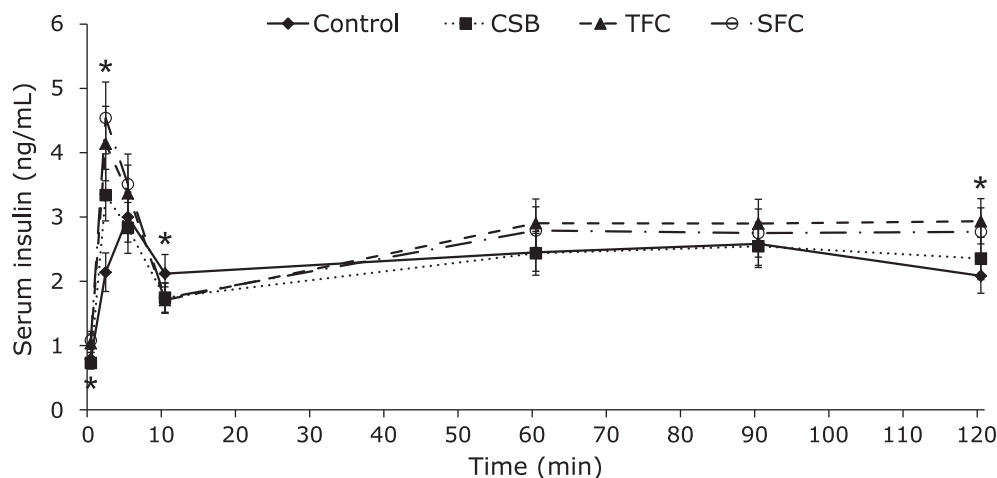
Fig. 2. Insulin secretion capacity during hyperglycemic clamp. At the end of the experimental periods, hyperglycemic clamp was performed on Px rats fed diets containing 10% cooked soybeans (CSB), chungkookjang made in the traditional (TFC) or standardized (SFC) manner, or casein (control). During hyperglycemic clamp, serum insulin levels were measured in free-moving and overnight-fasted diabetic rats as serum glucose levels at 5.5 mM above fasting levels were maintained. The sample size in each group was the same as in Table 3. *Significantly different among groups of Px rats at $p<0.05$.

Table 3. Insulin secretion capacity during hyperglycemic clamp

	Control (n = 10)	CSB (n = 10)	TFC (n = 10)	SFC (n = 10)	Normal-control (n = 10)
Serum insulin at basal state	0.79 ± 0.13^b	0.74 ± 0.13^b	1.07 ± 0.20^a	1.04 ± 0.19^a	$1.46 \pm 0.24^†$
Serum insulin at first phase (ng/mL)	2.57 ± 0.37^c	3.09 ± 0.38^b	3.76 ± 0.43^a	4.03 ± 0.49^a	$4.76 \pm 0.56^†$
Serum insulin at second phase (ng/mL)	2.37 ± 0.33^b	2.44 ± 0.34^b	2.91 ± 0.36^a	$2.77 \pm 0.33^{a,b}$	$4.02 \pm 0.58^†$
Area under the curve of insulin (AU)	41.7 ± 5.3^b	43.2 ± 5.6^b	50.5 ± 5.9^a	50.8 ± 6.3^a	$70.5 \pm 9.3^†$
Glucose infusion rate (mg/kg bw/min)	8.4 ± 1.3^c	11.5 ± 1.6	13.9 ± 1.6^a	13.5 ± 1.8^a	$29.3 \pm 3.5^†$
Insulin sensitivity (μmol glucose·min^{-1}·100 g^{-1} per μmol insulin/L)	$10.8 \pm 1. 4^b$	15.7 ± 1.9^a	15.1 ± 1.8^a	15.6 ± 1.9^a	$16.3 \pm 2.0^†$

Values are mean ± SD. First phase insulin secretion was defined as the average of serum insulin levels at 2 and 5 min, with second phase at 60, 90 and 120 min. Insulin sensitivity at hyperglycemic state was calculated as the ratio of glucose infusion rates to steady-state serum insulin levels. Diabetic rats in the control, CSB, TCF and SFC groups were provided with 40 En% fat diets added with casein, 10% cooked soybean, chungkookjang made with traditional manner and chungkookjang made with standard manner, respectively. [a,b]Values in the same row with different superscripts were significantly different in Tukey's test at $p<0.05$. [†]The normal-control group of non-diabetic rats were significantly different from the control group of Px rats at $p<0.05$ by two sample t test.

Table 4. The modulation of islet morphometry

	Control (n = 7)	CSB (n = 7)	TFC (n = 7)	SFC (n = 7)	Normal-control (n = 7)
β-cell area (%)	6.6 ± 0.7^b	7.0 ± 0.8^b	7.9 ± 0.8^a	8.2 ± 0.9^a	$5.5 \pm 0.7^†$
Individual β-cell size (μm^2)	239.4 ± 28.4^a	198.3 ± 24.2^b	188.5 ± 26.8^b	185.4 ± 27.5^b	$185.6 \pm 23.2^†$
Absolute β-cell mass (mg)	21.8 ± 2.7^b	23.1 ± 3.0^b	26.0 ± 3.0^a	27.1 ± 3.3^a	$34.8 \pm 4.4^†$
BrdU$^+$ cells (% BrdU$^+$ cells of islets)	0.84 ± 0.10^b	0.89 ± 0.09^b	1.07 ± 0.11^a	1.11 ± 0.10^a	$0.72 \pm 0.10^†$
Apoptosis (% apoptotic bodies of islets)	0.74 ± 0.08^a	0.63 ± 0.08^b	0.63 ± 0.07^b	0.62 ± 0.07^b	0.65 ± 0.09
Ratio of β:α cells	4.7 ± 0.6^b	5.4 ± 0.6^a	5.7 ± 0.8^a	5.9 ± 0.7^a	$5.8 \pm 0.7^†$

Values are mean ± SD. Diabetic rats in the control, CSB, TCF and SFC groups were provided with 40 En% fat diets added with casein, 10% cooked soybean, chungkookjang made with traditional manner and chungkookjang made with standard manner, respectively. [a,b]Values in the same row with different superscripts were significantly different in Tukey's test at $p<0.05$. [†]The normal-control group of non-diabetic rats were significantly different from the control group of Px rats at $p<0.05$ by two sample t test.

comparison to the unfermented CSB, and the aglycones were increased the most in BL and TFC. Since isoflavonoid aglycones are better absorbed than isoflavonoid glycans in *in vivo*,[23] their bioavailability and bioactivity are expected to be increased in chungkookjang, especially when fermented with *B. lichemiformis*.

It was interesting that cooked soybeans as well as both chungkookjangs partially restored fasting serum glucose concen-

tration, but only the chungkoojangs increased fasting insulin levels. That trend was also seen in the insulin curve during hyperglycemic clamp and was explained by the greater β-cell mass and BrdU incorporation indicating increased proliferation of β-cells. These results suggest that bioactive compounds are present in the fermented soybeans that facilitate the regeneration of β-cells that are either not present in cooked soybeans or present at much lower
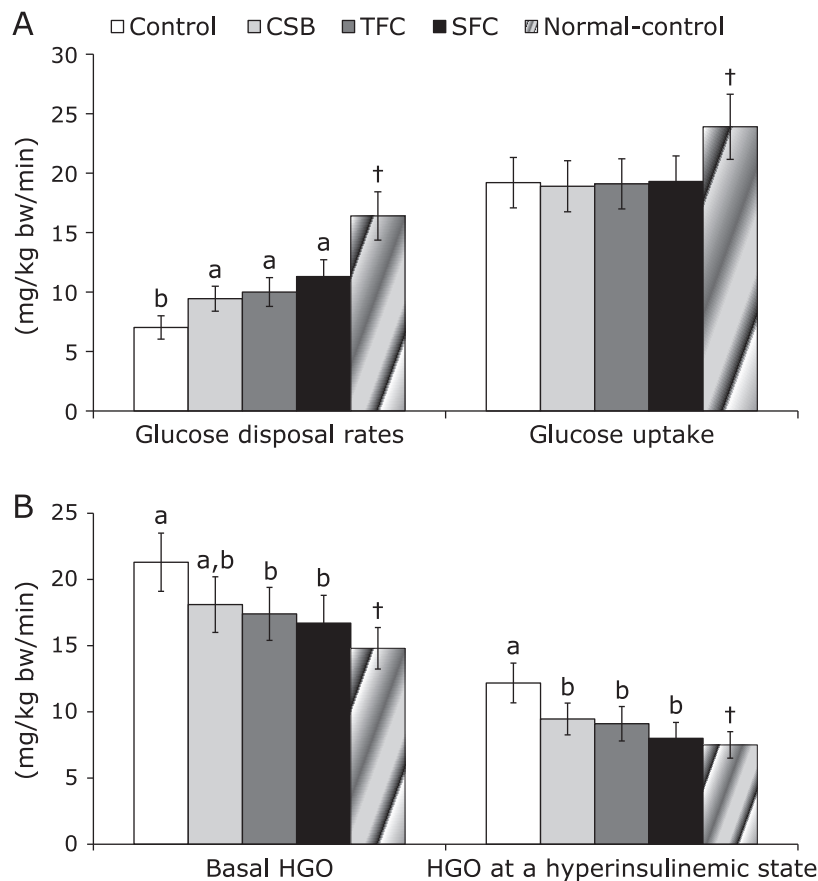
Fig. 3. Metabolic parameters during euglycemic hyperinsulinemic clamp. After 8 weeks of treatment with four different diets—one containing 10% cooked soybeans (CSB), one containing chungkookjang made in the traditional (TFC), one containing chungkookjang made in the standardized (SFC) manner, and one containing casein (control)—euglycemic hyperinsulinemic clamp was performed in conscious, free moving and overnight fasted diabetic rats to determine whole body insulin resistance. Whole body glucose infusion rates (GIR) and glucose uptake (A) and hepatic glucose output at basal and clamped states (B) were investigated. The sample size in each group was the same as in Table 3. [a,b]Values in the same row with different superscripts were significantly different in Tukey's test at $p < 0.05$. [†]Significantly different from Px control at $p < 0.05$.

levels. They are possibly isoflavonoid aglycones such as diadzein and genistein, and small peptides.[24–27] Especially genistein has shown to improve glucose-stimulated insulin secretory capacity and β-cell viability in insulinoma cells. [24] In addition, genistein has been reported to prevent β-cell loss via potentiating β-cell proliferation in diabetic animals.[25–27]

Daidzein as well as genistein improved glucose homeostasis via improving insulin sensitizing activity.[11] In the present study, insulin signaling in the liver appeared to be improved more by the SFC than even traditional chungkookjang since phosphorylated Akt was almost 3 fold higher in the livers of rats following hyperglycemic clamp and that was supported by modestly higher pAMPK and lower PEPCK. This related to the attenuation of insulin resistance in the liver. The improvement of insulin resistance was related to isoflavonoids and small peptides. Consistent with our study, Choi *et al.*[11] reported that geneistein and daidzein prevented hyperglycemia via reducing hepatic gluconeogenesis and lipogenesis. *In vitro* studies have shown that daidzein exerts its insulin sensitizing actions in 3T3-L1 adipocytes via activating PPAR-γ, the central regulator of insulin and glucose metabolism,[24,28,29] although the enhancement was less than rosiglitazone, a commercial PPAR-γ agonist. In addition, 1–1.5 kDa peptide profiles from chungkookjangs fermented with *B. lichemiformis*, but not *B. subtilis* and *B. amylofenices*, were similar to those of TFC and appeared to be associated with improved insulin sensitivity. Furthermore, we previously demonstrated that peptides

greater than 3 kDa do not contribute to the anti-diabetic activity of fermented soybeans.[30] Therefore, it is reasonable to conclude that the standardized chungkookjang was at least as protective against type 2 diabetes in diabetic rats as its traditional counterpart, and possibly more protective.

Since degenerative diseases such as type 2 diabetes increase worldwide as traditional diets undergo rapid changes or are abandoned altogether, it must be questioned if protective factors in those diets are being lost, resulting in an increased incidence of diseases. It is unlikely that any one component of a traditional diet could provide protection from a disease such as type 2 diabetes, but it has been demonstrated in Inuit groups that individuals who abandon a traditional diet in favor of a Western diet have a greater incidence of diabetes than those who remain on the traditional diet.[31] Likewise, it is unlikely that simply eating typical amounts of either chungkookjang would protect against type 2 diabetes, but the aggregate of many changes to the traditional Korean diet might indeed increase the risk of metabolic diseases such as obesity and diabetes. It is probably not possible, and possibly undesirable to prevent changes to modern diets; however, it is possible to design foods that retain the functional benefits of their traditional counterparts. Traditionally made chungkookjang is difficult make with consistent functional efficacy due to dependence on local environment. Thus, it is important to make chungkookjang using methods that can be controlled and replicable. Thus, it is necessary to find the right microorganism to
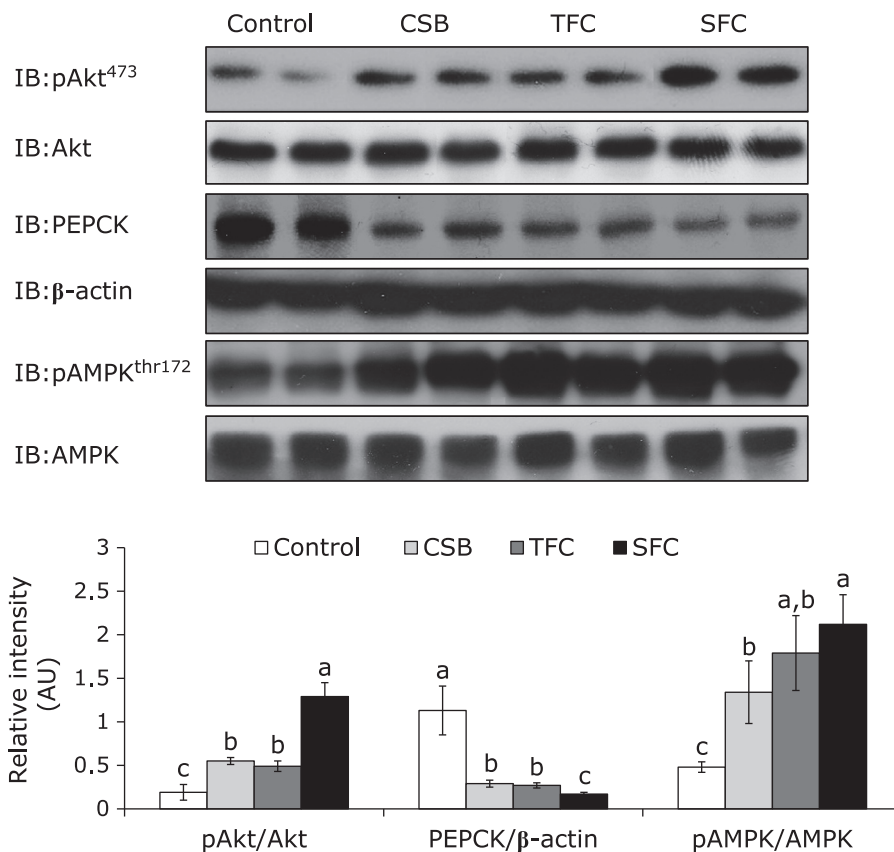
Fig. 4. Hepatic insulin signaling at the end of experiment. The liver of Px rats injected with 5 U insulin/kg body weight into the inferior vena cava was collected after they were fed with diets containing 10% cooked soybeans (CSB), chungkookjang made with traditional (TFC) or standardized (SFC) manner, or casein (control) for 8 weeks. The phosphorylation of Akt and AMPK and the expression of phosphoenolpyruvate carboxykinase (PECK) were measured by immunoblotting method. The sample size in each group was 4. [a,b]Values in the same row with different superscripts were significantly different in Tukey's test at $p<0.05$.

ferment soybeans to make standardized chungkookjang having anti-diabetic action. Chungkookjang fermented with *B. lichemifomis* for 48 h has equal or better anti-diabetic action than traditionally made chungkookjang.

In conclusion, *B. licheniformis* may be an important microorganism for maximizing the anti-diabetic actions of soybeans during fermentation of chungkookjang, and may be useful for producing commercial chungkookjang with consistent anti-diabetic activity. This research demonstrated that a standardized commercial chungkookjang made with *B. licheniformis* can exert equal or superior insulinotropic and insulin sensitive activity to that of the best quality traditionally produced TFC in diabetic rats.

Acknowledgments

Conflict of Interest

The authors declare no conflicts of interest that are relevant to this article.

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