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Raw vegetable food containing high cyclo (his-pro) improved insulin sensitivity and body weight control

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Abstract

Cyclo (his-pro), controlled-energy diet, soy protein hydrolysate (SPH), and raw vegetable food (RVF) are known to improve insulin sensitivity and body weight (BW) control. Enhancement of high cyclo (his-pro) content in SPH (HCS) was performed by refluxing SPH with 1 N KH_2CO_3 dissolved in 70% ethanol for 2 weeks at room temperature. Using this material, we examined the effects of HCS plus RVF on glucose metabolism and BW control in genetically diabetic Goto-Kakizaki (G-K) and insulin-resistant aged overweight Sprague-Dawley (S-D) rats. Thirty 7-week-old G-K rats and 18 16- to 18-month-old S-D rats were divided into 3 groups and treated with normal chow (NC), RVF diet, or HCS diet for 8 weeks. Raw vegetable food diet was made of 1:3 RVF and 2:3 NC; HCS diet was made of 1:27 portion HCS, 8:27 RVF, and 2:3 NC. Oral glucose tolerance significantly improved in both RVF- ($P < .01$) and HCS-treated ($P < .001$) G-K rats and worsened in NC-fed rats compared with the baseline values. Similarly, oral glucose tolerance also improved in aged overweight S-D rats when treated with RVF ($P < .05$) and with HCS ($P < .01$), compared with the baseline values. Although HCS diet treatment very significantly lowered fed plasma insulin levels compared with NC diet treatment in G-K rats ($P < .01$), RVF diet treatment alone did not decrease plasma insulin levels. In contrast, there was no change of insulin levels in overweight aged S-D rats after either RVF or HCS diet treatment. Postfeeding glucose levels in G-K rats fed RVF or HCS significantly fell, compared with the rats fed NC ($P < .05$). Interestingly, fasting blood glucose levels in RVF- or HCS-fed rats were very significantly lower than in NC-fed rats ($P < .001$). There was no change of blood glucose levels in S-D rats due to treatments with different diet. In G-K rats, food intake did not decrease during the first 3 weeks but fell very significantly from the fifth to eighth weeks with RVF ($P < .01$) and HCS ($P < .001$) treatments in G-K rats. However, food intake reduction in aged S-D rats was shown only for the HCS-treated rat group ($P < .05$). Water intake slightly decreased in G-K rats with either RVF or HCS treatment ($P < .05$) but very significantly decreased in S-D rats with HCS treatment ($P < .01$). Body weight gain in young G-K rats and BW in aged S-D rats significantly decreased only when rats were treated with HCS diet ($P < .05$). These data suggest that regular consumption of HCS diet helps to control blood glucose metabolism in diabetic G-K rats and BW control in aged obese S-D rats.

1. Introduction

Overweight and obesity from hyperphagia and high-fat diet are the major causes of insulin resistance [1,2]. It is well established that an energy-restricted diet and exercise are the most desirable treatments for diabetes and obesity. The most effective dietary management for the control of diabetes and obesity is to maintain a negative energy balance for a period. This treatment results in increased insulin sensitivity [3]. Thus, an energy-restricted diet is highly desirable for the control of diabetes and obesity.

Large differences have been observed with different starch-containing foods in the control of blood glucose because cooking starch-containing food increases glucose

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about 50%. Thus, it has been reported that raw starch ingestion resulted in a 44% lower glucose response and a 35% to 65% lower insulin response compared with either glucose or sucrose ingestion [4]. Foods such as rice and potato release more glucose in relation to the time of cooking [5], but uncooked legumes, which have more indigestible starch, are ideal foods for the control of diabetes [6]. Patients who consumed whole-grain in a long-term cohort study had reduced risk of type 2 diabetes compared with controls who consumed white rice. These studies suggest that consumption of an energy-restricted diet consisting of raw vegetable food (RVF) would reduce the incidence and delay the development of diabetes. However, a cohort study using RVF for the treatment of diabetes has not been established.

Although soy proteins are deficient in methionine, an essential amino acid, soy protein–containing diets exhibit protective effects against diabetes and obesity [7]. Natural rat chow with high carbohydrate and meat substances has been shown to promote diabetes, as opposed to a casein-based, defined diet which was shown to significantly inhibit development of age and obesity-induced diabetes [8]. Thus, soy protein intake ameliorates insulin resistance and glycemic control in type 2 diabetes. One of the possible mechanisms by which soy proteins affect diabetes is through the activity of isoflavones. Isoflavones may activate peroxisome proliferator–activated receptors (PPAR) which can enhance insulin action [9-12].

We recently discovered that cyclo (his-pro) (CHP), a cyclic form of L-histidyl-proline, is present in large quantities in ethyl alcohol-refluxed soy protein hydrolysate (SPH). It has a strong ability to stimulate intestinal zinc absorption, cellular zinc uptake, and glucose utilization [13-15]. Dietary feeding of CHP plus zinc significantly improved insulin sensitivity and glucose clearance in (1) genetically type 2 diabetic Goto-Kakizaki (G-K) rats; (2) insulin-resistant, obese aged Sprague-Dawley (S-D) rats [15]; (3) streptozotocin-induced type 1 diabetic rats [14]; and (4) genetically type 2 diabetic obese ob/ob mice [16]. Inhibition of dipeptidase, which degrades CHP and/or its precursor, L-histidyl-proline, improves glucose tolerance in mice [17,18]. Cyclo (his-pro) also decreases food intake [19,20], and in that way, mimics the action of leptin, which controls appetite. Thus, CHP activity is closely related to insulin and leptin sensitivities, and impairment in the activities of these peptides may partly contribute to the development of diabetes and obesity.

Although exact mechanisms of antidiabetes and anti-obesity activities of CHP are not clearly established, it is highly likely that CHP is involved in the regulation of insulin and leptin sensitivity by stimulating zinc metabolism because of the following arguments: oral intake of CHP plus zinc significantly improves oral glucose tolerance (OGT) in diabetic and overweight animals in the state of decreased or unchanged insulin levels [15,16]; high CHP concentration inhibits insulin and glucagon secretion from islet cells in vitro [21], and CHP stimulates zinc transport mechanisms across the small intestine and cell membrane of muscle cells to increase zinc use [13]. Thus, these arguments suggest that CHP plays an important role in regulating insulin sensitivity via stimulation of zinc absorption and metabolism.

Zinc is involved in gene expression of glucose transporter 4 (GLUT4) [22], is an integral part of the insulin degrading enzyme [23,24], and stimulates insulin receptor β-subunit autophosphorylation [25,26]. Zinc also increases cellular glucose uptake [14]. However, zinc levels in plasma and cells of diabetic animals and humans are low, partly because of hyperzincuria [27-34]. Furthermore, intestinal zinc absorption rate is decreased in these subjects [35-39]. Epidemiologic studies have indicated that prevalence of diabetes and glucose intolerance is significantly higher among subjects consuming lower dietary zinc [40,41]. Although impaired glucose tolerance was improved with 3-time doses of 200 mg zinc sulfate per day, high physiological doses of zinc were minimally effective in the control of blood glucose [14,42-44]. Therefore, it appears that the principal role of CHP is to improve insulin resistance via modulating zinc metabolism [13].

Based on the these studies [1-44], we hypothesized that improving zinc metabolism by treating animals with controlled-energy diet containing RVF and high CHP-containing SPH (HCS) improves glucose metabolism to prevent and treat mammalian diabetes and obesity. To test this hypothesis, we determined the effects of RVF and HCS on glucose metabolism and body weight (BW) control in genetically type 2 diabetic G-K and insulin-resistant, aged overweight S-D rats.

2. Materials and methods

2.1. Materials

2.1.1. Animals

Five 1-month-old male and 5 age-matched female G-K rats were purchased from the University of South Florida, Comparative Medicine Department (Dr Robert V. Farese), and the colony was expanded at the animal facility of the VA Greater Los Angeles Healthcare System, Los Angeles, Calif. Goto-Kakizaki rats were used at 5 to 8 weeks of age. These rats are genetically type 2 diabetic and express insulin resistance at birth. About 1-year-old retired breeder S-D rats were purchased from Charles River Laboratories, Indianapolis, Ind, and maintained in the Animal Facility of the VA Greater Los Angeles Healthcare System, Los Angeles, Calif. These studies were conducted with the approval of the Institutional Animal Care and Animal Use Committee of the VA Greater Los Angeles Healthcare System.

2.1.2. Reagents and chemicals

RIA kit for insulin assay was purchased from LINCO Research, Inc, St Charles, Mo. Ethyl alcohol and $KHCO_3$ were purchased from Sigma Co, St Louis, Mo.

Table 1
Ingredient list of RVF

Food sources	Amount (%)	Functional ingredients
Brown rice	19.45	Resistant starch
Sprouted brown rice	10.42	Resistant starch, intact enzymes
Corn	6.85	Carbohydrates, resistant starch essential fatty acid
Glutinous millet	1.39	Carbohydrate, fiber, proteins
Sorghum	1.49	Carbohydrate, fiber, proteins
Pearl barley	1.09	Fructofuranose (inulin), rutin, favorable amino acids
Soybean	1.49	Proteins, isoflavones
Black sesame	0.45	Proteins, essential fatty acids
Carrot	1.19	β-Carotene, α-carotene, fiber
Burdock	0.79	Polyacetylenes
Kale	7.84	Fiber
Pine needle	0.006	Flavor, mineral enzyme activities
Pumpkin	24.30	Fiber
Radish root	2.03	Bioflavonoids, indoles, potassium
Radish leaves	0.89	Fiber, vitamin C
Mugwort	0.06	Flavor, camphor, thujone
Angelica utilis	0.13	Functional ingredients not defined
Lentinus edodes	0.09	Lentinan, neutral polysaccharide, essential fatty acids
Ganoderma lucidum	0.025	Triterpenes, ganodermic acid.
Laver	0.13	Essential fatty acids, minerals
Brown seaweed	0.19	Essential fatty acids, minerals
Tangle	0.26	Essential fatty acids, minerals
Royal jelly	0.06	Minerals, vitamins.
Red ginseng	0.02	Triterpene, lactobacillus, saponin
Fructooligosaccharide	13.80	Fructose
Spirulina	0.23	Essential fatty, *Mortierella alpina*, arachidonic acid
Cabbage	2.43	Vitamin C, indole
Green tea extract	0.23	Trieterpene, saponin
Soy protein	2.06	Isoflavone
Soy protein peptide	0.27	Amino acids
Buck wheat	0.33	Rutin (flavonoids)

2.1.3. Experimental diets

Normal chow (NC) (PROLAB RMH 2500) was purchased from PMI Nutrition International, LLC, Brentwood, Mo. Raw vegetable food was provided by Juvo Inc, Buena Park, Calif. Table 1 includes an ingredient list for RVF. This RVF diet contains 6.25% fat, 75% carbohydrate, 10% protein, 2.5% fiber, 5% sugar, and 1.25% minerals. Soy protein hydrolysate was purchased from Marco Development Co, Carlstadt, NJ. HCS was made by dissolving SPH in 70% ethyl alcohol containing 1 N $KHCO_3$ and refluxed at 25°C for 2 weeks to increase CHP content, as described previously [45]. The final HCS preparation contains 1 g zinc per kilogram of HCS. Normal chow contains 17.01 kJ/g, RVF 0.714 kJ/g, and SPH 16.8 kJ/g. RVF-HCS is a mixture of 1 part HCS and 8 parts of RVF, and the zinc concentration in this mixture is 111 mg/kg, which is about twice the zinc concentration in the same amount of NC. To prevent the taste aversion effects of RVF or RVF-HCS diets on the food intake, all the experimental diets, including control diet, were reinforced with 2.5% of Equal sweetener (wt/wt).

2.2. Methods

2.2.1. Experimental diet feeding procedures

Thirty G-K rats (7 weeks old) and 18 aged S-D rats (16 to18 months old) were divided into 3 groups (10 or 6 rats per group) and were kept on exclusively experimental diets for 3 to 12 weeks. All 3 experimental diets were mixed 1:1 wt/vol with water to make the diets gooey. The first group of rats were treated with NC, the second group with RVF diet (contains 2:3 NC and 1:3 RVF powder [wt/wt]) and the third group with RVF-HCS diet (contains 2:3 NC and 8:27 RVF and 1:27 HCS). Raw vegetable food diet contains 11.592 kJ/g and RVF-HCS diet, 12.18 kJ/g. These foods were provided in a 300-mL-capacity stainless steel bowl with a cover that has a 1.5-cm-diameter hole to allow rat access to the foods without spilling foods from the container. The amount of food remaining was measured every other day to determine each rat's food and energy intake.

2.2.2. Measurements of OGT and plasma insulin levels

Blood glucose was measured by Accu-Chek Glucometer (Roche Diagnostics Corp, Indianapolis, Ind) with a sample obtained from a cut at the end of each rat's tail. Three-hour-area-above-fasting-blood glucose concentrations (TAFGC), an index of OGT, was measured by measuring blood glucose every 30 minutes for 3 hours after gastric gavage of 1.0 g glucose per kilogram of BW to overnight-fasted rats. TAFGC value for each animal was determined by subtracting each animal's fasting glucose level from the values obtained for each time point. The differences were then averaged across all times (0-3 hours), and the average value is TAFGC. At the end of diet treatment, rats were anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg), and after which, heparin was infused through the tail vein. Rats were exsanguinated through the ocular vein. Plasma was collected by centrifugation of blood and frozen at −80°C until analysis. Plasma or serum insulin concentrations were measured by RIA kit.

2.2.3. Measurements of fed blood glucose levels, food intake, and water intake

Blood glucose levels were measured every other day during the experimental period, and the rate of decrease in blood glucose was calculated by regression analysis for each rat. Average water intake was determined by dividing the total amount of water consumed in each rat group by total rat weight and days. Average food intake was measured by determining the amount of food decrease in the food container divided by rat weight and days. Body weight was measured every day and regression analysis for the change in each rat was determined during each experimental diet feeding period.

2.2.4. Measurements of blood chemical parameters for liver and kidney functions

Liver and kidney panels were determined at a commercial laboratory using an automated blood chemical analyzer (Vet-West Lab, Van Nuys, Calif).

2.2.5. Statistical analysis

Analysis of variance and paired or unpaired *t* tests were carried out using a GraphPad InStat, version 5.0, supplied by GraphPad Software Co, San Diego, Calif. A *P* value of less than .05 was considered statistically significant.

3. Results

3.1. Oral glucose tolerance test

As shown in Fig. 1A, G-K rats treated with RVF or RVF + HCS had significantly lower TAFGC value compared with those treated with NC. TAFGC value in rats treated with RVF + HCS was more significantly improved than in rats treated with RVF alone ($P < .01$ vs $P < .05$). In obese S-D rats, both RVF and RVF + HCS treatments similarly decreased TAFGC values compared with that of controls. *P* value in RVF + HCS was greater than in rats treated with RVF only ($P < .01$ vs $P < .05$) (Fig. 1B).

3.2. Plasma insulin levels

Plasma insulin levels significantly decreased only in G-K rats treated with RVF + HCS ($P < .01$), but no difference was observed between rats treated with RVF alone and control rats (Fig. 2A). However, plasma insulin levels were normal and did not change because of any of the dietary treatments in aged overweight S-D rats (Fig. 2B).

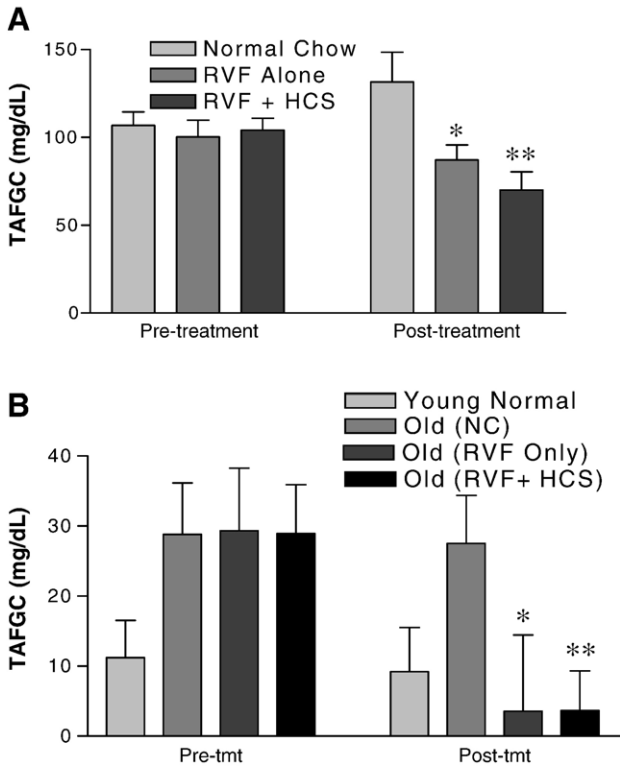

Fig. 1. Oral glucose tolerance tests expressed by TAFGC. A, TAFGC values in 7-week-old genetically diabetic G-K rats. B, TAFGC values in aged S-D rats. Values are mean ± SEM. (n = 10 for G-K rats and n = 6 for aged S-D rats). Post-treatment values were compared with the pretreatment values after 8-week treatments. *$P < .05$; ** $P < .01$ vs NC.

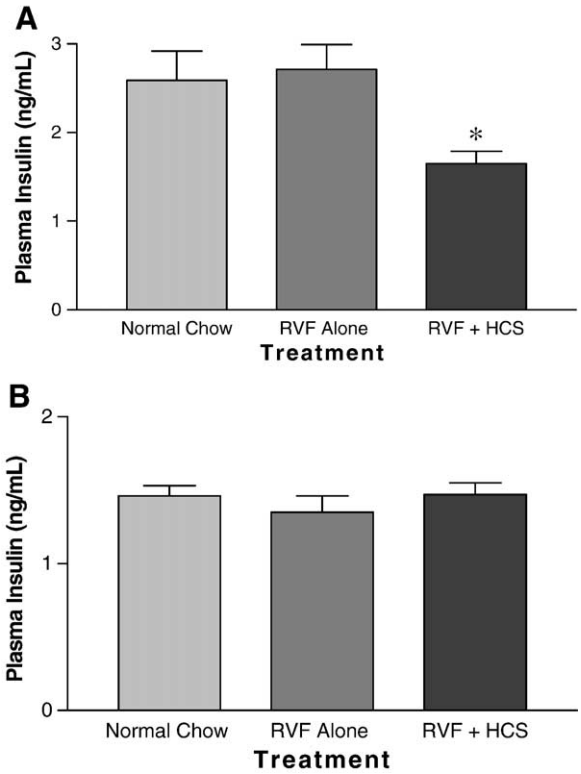

Fig. 2. Plasma insulin levels. A, Plasma insulin levels in 7-week-old genetically diabetic G-K rats. B, Plasma insulin values in aged S-D rats. All the rats were treated with different diets for 8 weeks, and plasma insulin levels were compared with the levels in rats treated with NC. Values are mean ± SEM (n = 10 for G-K rats and n = 6 for aged S-D rats). *$P < .01$ vs NC.

3.3. Blood glucose levels

Both RVF- and RVF + HCS–treated rats showed moderately low-fed blood glucose levels, compared with NC-treated G-K rats ($P < .05$) (Fig. 3A). In contrast, fasting venous blood glucose levels were dramatically low in G-K rats treated with RVF and RVF + HCS, compared with rats given NC ($P < .001$) (Fig. 3B). However, blood glucose levels were normal, and no difference was shown regardless of different dietary treatments (NC, RVF, or RVF + HCS) in aged nondiabetic S-D rats.

3.4. Body weight gain in young G-K rats and BW loss in aged S-D rats

Although BW gain similarly decreased in both RVF- and RVF + HCS–treated 7 week-old G-K rats, only rats treated with RVF + HCS rats showed statistical significance in BW gain ($P < .05$) (Fig. 4A). When aged overweight S-D rats were treated with RVF or RVF + HCS diet, BWs in rats treated with RVF + HCS significantly decreased, compared with the pretreatment weights ($P < .05$) (Fig. 4B).

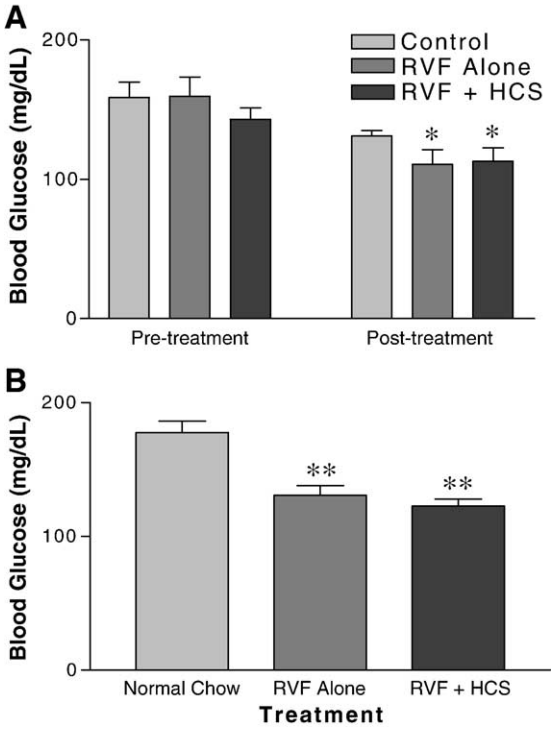

Fig. 3. Fed and fasting blood glucose levels in G-K rats. A, Fed blood glucose levels of 7-week-old genetically diabetic G-K rats before and after the treatment with experimental diets for 8 weeks. B, Fasting blood glucose levels in these rats. Blood glucose concentrations were measured at baseline and after treatment with NC, RVF, or RVF + HCS diet for 8 weeks. Values are mean ± SEM. *$P < .05$; **$P < .001$ vs NC.

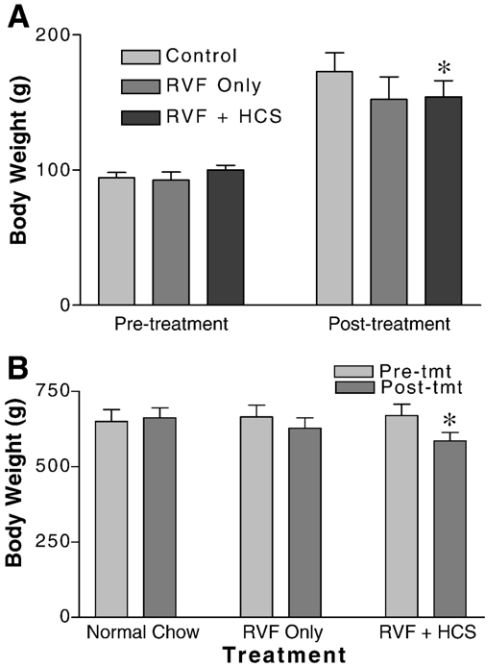

Fig. 4. Body weight gain in young G-K rats (A) and body weight loss in aged obese S-D rats (B). Body weight gain after the treatment with RVF + HCS in 7-week-old G-K rats was significantly lower in rats treated with RVF-HCS compared with NC-treated rats. Body weight after the treatment with RVF + HCS also significantly decreased in aged obese S-D rats. *$P < .05$ vs NC.

However, the decrease of BWs in rats treated with RVF alone did not reach statistical significance.

3.4.1. Food intake

Food intake in G-K rats did not change among all 3 rat groups during the first 3 weeks. However, during the last 3 weeks, food intake rate was dramatically lower for those given RVF ($P < .01$) or RVF + HCS ($P < .001$), compared with controls (Fig. 5A). However, there was no significant decrease in food intake in RVF diet–fed rats in aged S-D rats, but RVF + HCS diet–fed rats significantly decreased food intake ($P < .05$) (Fig. 5B).

3.4.2. Energy intake

Energy intake in G-K rats was very significantly lowered in both RVF alone– and RVF +HCS–treated rat groups during the entire 8-week treatment periods in both G-K and aged obese S-D rats ($P < .001$ for G-K rats and $P < .01$ for S-D rats, respectively), compared with controls (Fig. 6A and B). However, energy intakes did not significantly differ between rats treated with RVF alone and those with RVF + HCS. Reduction of energy intake in either RVF- or RVF + HCS–treated G-K or aged S-D rats were very similar to eating the same volume of food that had lower energy density.

3.4.3. Water intake

Water intake significantly decreased in either RVF- or RVF + HCS–treated G-K rats compared with NC-treated rats ($P < .05$) (Fig. 7A). Both RVF and RVF + HCS

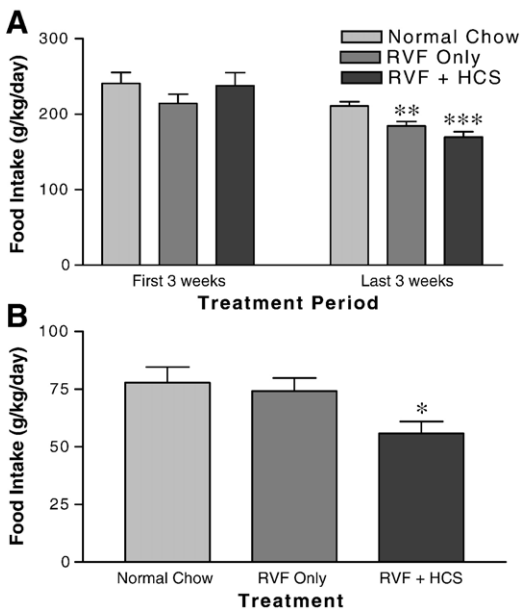

Fig. 5. Food intakes in G-K and S-D rats. Food intakes in G-K rats were measured during the 8-week treatment period in 3 groups of rats treated with NC, RVF, or RVF + HCS (A). No difference was shown during the first 3 weeks of treatments, but both RVF and RVF + HCS diet treatment significantly decreased food intake during the last 3 weeks. However, only rats treated with RVF + HCS decreased food intake in aged obese S-D rats (B). *$P < .05$ vs NC; **$P < .01$ with RVF; ***$P < .001$ with RVF +SPH vs NC.

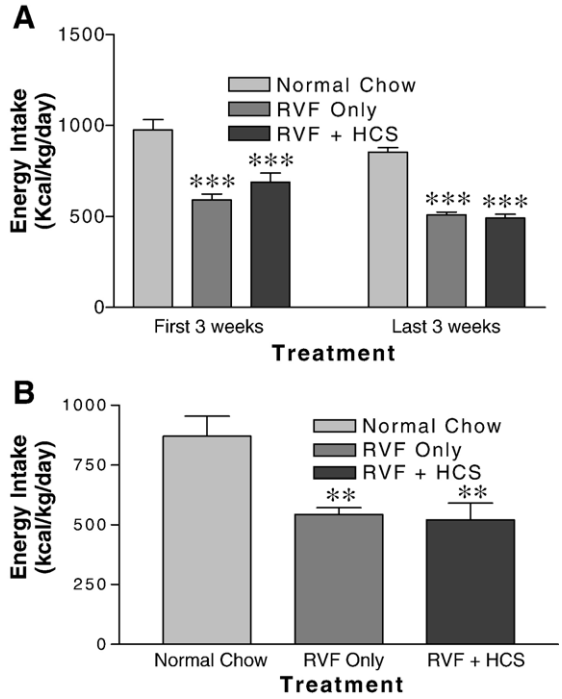

Fig. 6. Energy intakes in G-K and S-D rats. Energy intakes in G-K rats were measured during the first 3 weeks and the last 3 weeks in the 8-week treatment period in 3 groups of rats treated with NC, RVF, or RVF + HCS (A). Energy intakes were significantly low during the entire 8-week treatments in RVF- and RVF + HCS–treated G-K and obese S-D rats compared with controls (***P < .001 for G-K rats and **P < .01 for S-D rats vs NC).

treatments in aged S-D rats also significantly decreased water intake compared with NC-treated rats (P < .05 and .01, respectively) (Fig. 7B). However, HCS addition to RVF diet led to more dramatically reduced water intake in aged S-D rats than those treated with RVF alone. Thus, adding HCS may have some effect in water intake in relation to obesity rather than to diabetes.

3.4.4. Blood chemistry data

In general, chemistry data showed improved clinically relevant biochemical abnormalities (activities of alkaline phosphates, alanine aminotransferase [ALT], total bilirubin [Tbi], blood urea nitrogen (BUN), potassium, glucose, creatinine, phosphate, aspirate aminotransferase, and γ-glutamyl transferase [γ-GT]) in rats treated with RVF alone compared with NC-treated controls and greater improvement in those treated with RVF + HCS diet (Table 2). However, there was essentially no change in liver and kidney panels in aged S-D rats treated with different diets (Table 3). Although other data are involved with certain disease or side effect, only the chemicals changed with RVF or RVF + HCS explain the meaning of changes as follows:

1. *Serum alkaline phosphatase activity* in both G-K and aged obese S-D rats significantly decreased with RVF + HCS treatment. Serum alkaline phosphatase activity reflects a group of isoenzymes derived from liver, bone, intestine, and placenta. Thus, elevation of this enzyme is associated with a variety of clinical conditions such as hepatocellular diseases, cholestasis, partial or complete bile duct obstruction, and infiltrative liver disease such as neoplasm, granuloma, and bone destruction. Deceased alkaline phosphatase with the RVF + HCS may reflect improvement or prevention of these clinical conditions. This phenomenon shows that RVF + HCS intake is safe and beneficial.
2. *Alanine aminotransferase activity* is an intracellular amino-transferring enzyme present in large quantities in hepatocytes. It is released into the circulation. Thus, serum levels of this enzyme are a sensitive test of liver damage such as hepatocellular necrosis, fatty liver caused by obesity, or diabetes. Our results suggest that the levels of this enzyme in both RVF + HCS–treated G-K and obese rats are lower than in those treated with only RVF, suggesting that RVF + HCS intake is beneficial for the improvement of hepatic function in both diabetic and obese rats.

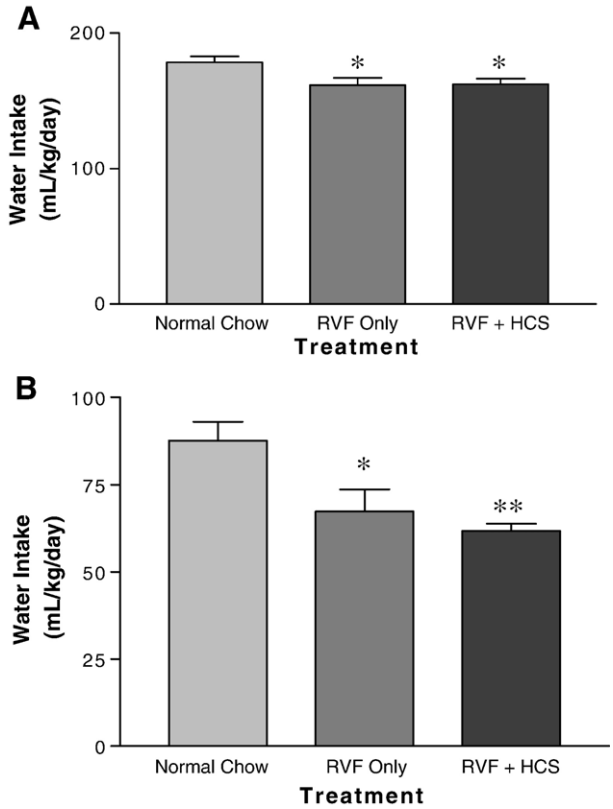

Fig. 7. Water intake in G-K and S-D rats. Treatments with RVF or RVF + HCS similarly decreased water intake in G-K rats (P < .05 vs NC) (Fig. 6A). However, in S-D rats, RVF + HCS treatment more significantly decreased water intake than RVF-only treatment (Fig. 6B) (**P < .01 and *P < .05 vs NC).

Table 2
Effects of RVF-HCS on blood biochemical concentrations related to liver and kidney functions in G-K rats

Chemicals (U)	Treatments			
	Controls (mean ± SEM)	RVF only (mean ± SEM)	RVF-HCS (mean ± SEM)	Reference range
AP (IU/L)	229.0 ± 25.6	194.5 ± 19.2	164.5 ± 28.5 ↓ (1)	38-126
ALT (IU/L)	52.6 ± 15.5	60.8 ± 11.4	43.5 ± 15.0 ↓ (2)	10-40
ALB (g/dL)	1.92 ± 0.03	1.85 ± 0.10	2.03 ± 0.08	3.0-4.5
TBP (g/dL)	6.27 ± 0.25	5.63 ± 0.23	6.10 ± 0.25	6.3-8.2
TBi (mg/dL)	0.95 ± 0.25	0.73 ± 0.05	0.58 ± 0.05 ↓ (3)	0.2-1.2
BUN (mg/dL)	12.7 ± 1.09	9.50 ± 2.02	13.0 ± 2.16 (4)	4-15
Na (mmol/L)	133.3 ± 6.0	125.8 ± 5.9	134.0 ± 5.4	130-145
K (mmol/L)	6.3 ± 0.5	5.6 ± 0.1	5.1 ± 0.4	3.5-5.5
Cl (mmol/L)	101.6 ± 3.3	94.8 ± 4.1	101.3.± 4.3	95-110
CO_2 (mmol/L)	19.0 ± 1.1	19.5 ± 1.6	20.5 ± 0.6	22-31
Glucose (mg/dL)	173.03 ± 5.1	166.3 ± 11.1	157.5 ± 18.4 ↓ (5)	65-105
Creatinine (mmol/L)	0.40 ± 0.03	0.35 ± 0.06 ↓	0.48 ± 0.03 ↑ (6)	0.8-1.5
Ca (mg/dL)	9.5 ± 0.3	9.1 ± 0.4	9.6 ± 0.4	8.4-10.2
PO_4 (mg/dL)	7.9 ± 1.0	5.7 ± 0.3	5.1 ± 0.3 ↓ (7)	2.5-4.5
AST (IU/L)	97.7 ± 6.0	77.0 ± 5.9	80.0 ± 17.2 ↓ (8)	20-57
γ-GT (IU/L)	1.0 ±.0 ↓	1.5 ± 0.3	2.0 ± 0.4 ↑ (9)	8-37

AP indicates alkaline phosphatase; ALB = albumin; TBP = total blood protein.
Numbers in parentheses correspond to numbers in text.

3. *Total bilirubin* increases with jaundice. Jaundice is manifested with hemolysis, neonatal jaundice, hepatocelluar disease, gallstones, tumors of bile duct, and biliary strictures. Although there was no change in aged S-D rats, G-K rats demonstrated significantly decreased total bilirubin levels. Again, this phenomenon shows that RVF + HCS diet intake causes no hepatic damage but shows beneficial effect on diabetes.
4. *Blood urea nitrogen* is one measurement of glomerular filtration rate. Urea is the major product of protein metabolism, and its production reflects the dietary intake of protein as well as the protein catabolic rate. Independent of renal function, increased BUN is related to gastrointestinal hemorrhage, catabolic tissue state, sepsis, and obstructive uropathy. Although G-K rats were not affected, BUN levels decreased in HCS-treated, aged obese rats. Thus, HCS diet is not harmful for kidney function but improves it to a certain level.
5. *Decreased plasma glucose levels are related to the improvement of diabetes and insulin resistance*. Blood glucose levels in both diabetic G-K rats and insulin-resistant, aged obese S-D rats showed a tendency to decrease with RVF + HCS diet treatment. This phenomenon is the major benefit of RVF + HCS diet in controlling diabetes.
6. Creatinine is derived from the catabolism of creatine in skeletal muscle. The *serum levels of creatinine are elevated with renal disease and dehydration*. Creatinine levels in both G-K and S-D rats treated with RVF + HCS showed a tendency to be higher

Table 3
Effects of feeding of RVF-HCS on blood biochemical concentrations related to liver and kidney functions in obese rats

Chemicals (U)	Treatments				
	Young Wistar (mean ± SEM)	Aged controls (mean ± SEM)	RVF only (mean ± SEM)	RVF-HCS (mean ± SEM)	Reference range
ALP (IU/L)	252 ± 35.9	89.0 ± 16.5	81.6 ± 15.5	71.4 ± 19.3 ↓ (1)	38-126
ALT(IU/L)	54.6 ± 2.1	42.2 ± 13.3	67.0 ± 16.	56.0 ± 12.1 ↓ (2)	10-40
ALB (g/dL)	1.46 ± 0.02	62 ± 0.19	1.86 ± 0.26	1.86 ± 0.21	3.0-4.5
TBP(g/dL)	5.75 ± 0.07	6.2 ± 0.41	6.62 ± 0.22	6.70 ± 0.26	6.3-8.2
TBi (mg/dL)	0.40 ± 0.04	0.46 ± 0.05	0.70 ± 0.10	0.52 ± 0.14 ↑ (3)	0.2-1.2
BUN (mg/dL)	15.4 ± 0.40	15.8 ± 1.36	12.6 ± 2.40	10.4 ± 2.04 ↓ (4)	4-15
Na (mmol/L)	140.2 ± 0.58	138.4 ± 0.93	139.2 ± 0.7	142.0 ± 2.1	130-145
K (mmol/L)	7.63 ± 0.13	4.86 ± 0.07	5.86 ± 0.23	5.78 ± 0.36	3.5-5.5
Cl (mmol/L)	105.8 ± 0.49	104.8 ± 0.49	104.8 ± 1.07	105.6 ± 2.38	95-110
CO_2 (mmol/L)	22.8 ± 1.32	22.8 ± 0.80	24.2 ± 0.49	22.8 ± 1.74	22-31
Glucose (mg/dL)	143.0 ± 3.8	138.4 ± 16.1	130.0 ± 10.1	106.0 ± 16.7 ↓ (5)	65-105
Creatinine (mmol/L)	0.38 ± 0.03	0.48 ± 0.05	0.46 ± 0.04	0.50 ± 0.04 ↑ (6)	0.8-1.5
Ca (mg/dL)	10.52 ± 0.07	9.08 ± 0.28	9.64 ± 0.12	9.68 ± 0.26	8.4-10.2
PO_4 (mg/dL)	7.50 ± 1.72	4.28 ± 0.45	4.68 ± 0.4	4.92 ± 0.3 ↑ (7)	2.5-4.5
AST (IU/L)	122.4 ± 6.4	85.6 ± 5.2	76.0 ± 13.7	116.2 ± 23.9 ↑ (8)	20-57
γ-GT (IU/L)	1.40 ± 0.40	1.20 ± 0.49	1.0 ± 0.00	1.6 ± 0.24 ↑ (9)	8-37

than the controls or rats treated with RVF only. The slight elevation of creatinine levels are probably reduced water intake in these rats when treated with RVF + HCS, but the levels are well within the reference ranges.

7. *Increased plasma phosphate (PO_4) reflects impaired glomerular filtration in the kidney or decreased parathyroid activation or hypocalcemia*. In the present studies, phosphate levels significantly decreased in G-K rats but increased in aged S-D rats with RVF + HCS treatment than controls or those treated with RVF alone. Phosphate is important for high-energy transfer reaction. Thus, young animals have high plasma phosphate, whereas aged animals, low plasma phosphate. Thus, CHP plus zinc in HCS may be beneficial in the control of energy transfer reactions in aged rats without beneficial effects on young rats.
8. *Serum aspartate aminotransferase (AST) activity* in both G-K and S-D rats treated with RVF only decreased, but addition of HCS increased AST concentration compared with controls. This enzyme is elevated with acute hepatic cell injury. However, slightly increased AST with the HCS treatment may not be related to liver damage because the AST activity is well within the reference range.
9. *γ-Glutamyl transferase* is elevated in liver disease from a variety of causes. Alcohol ingestion also causes elevated γ-glutamyl transferase. HCS treatment slightly increased γ-glutamyl transferase in both G-K and S-D rats. However, these elevations are well within the normal levels.

4. Discussion

Diabetes and obesity are epidemics in the United States [46,47], and obesity and overweight are major contributors to the development of diabetes [1,2]. A stable BW is maintained by matching energy expenditure with energy intake. Thus, energy overload leads to the development of obesity and diabetes. Energy restriction even for a brief period (4-20 days) increases whole-body insulin sensitivity, which is important for the management of type 2 diabetes in humans [3,48]. It has been reported that large differences exist between raw or cooked food in the control of blood glucose in persons both with and without diabetes [49]. For example, eating a cooked potato will rapidly increase blood glucose levels more than eating a raw potato [50]. Low glycemic index diets have been attracting attention in the prevention [51] and treatment [52] of diabetes. Jenkins et al [53] recently reported that vegetarian diets produce very significant metabolic advantage for the prevention and treatment of type 2 diabetes and its complications.

In addition to the beneficial effects of RVF in energy restriction to reduce energy intake, adding HCS into the RVF to increase CHP content significantly increased the effectiveness in the control of diabetes and insulin resistance compared with RVF alone (Figs. 1-3). Soy protein is becoming a more important component in the human diet, as it exhibits protective effects against obesity and diabetes [54]. The active ingredient in soy protein that improves diabetes by activating PPARγ has been identified as isoflavones [11]. Thus, soy consumption alleviates some of the symptoms associated with type 2 diabetes and insulin resistance [9,10]. Recently developed antidiabetic drugs for treating type 2 diabetic patients include Rosiglitazone [55,56] and Pioglitazone [57,58], which stimulate PPAR and thereby improve insulin resistance and diabetes. Soy products may work through a similar pathway to prevent and treat diabetes.

Soy protein hydrolysate, which contains amino acids, dipeptides, and other small peptides, is a better antidiabetes agent than soy protein or casein because SPH increases postprandial energy expenditure [59]. It is not surprising that spontaneous diabetes in the nonobese diabetic mouse was prevented by a soy protein–based infant formula, in which the protein source was replaced with soy casein hydrolysate [60]. Cyclo (his-pro) plus zinc has already been demonstrated to be effective in the prevention and treatment of insulin resistance in the type 2 diabetic G-K rats, aged obese S-D rats [15], and type 2 diabetic obese ob/ob mice [16]. We found in the present study that RVF + HCS with enhanced CHP concentration improves OGT in G-K rats (Fig. 1) better than RVF alone ($P < .01$ vs $P < .05$). Because all the diets were reinforced with artificial sweetener, these effects are not due to a taste aversion. For example, food intake in G-K rats during the first 3 weeks did not decrease, but it decreased after 5-week treatment with the experimental diets (Fig. 5A). Thus, the effects of RVF or RVF + HCS diets are due to physiochemical changes rather than taste aversion. Thus, our data show that energy-restricted diet (RVF) and HCS may play synergistic roles in the prevention and treatment of human diabetes.

Zinc is important in modulating insulin sensitivity by stimulating insulin receptor β-subunit autophosphorylation [25,26] and by being an integral part of insulin-degrading enzyme [61-63], which removes inactive insulin fragments from the cells and provides amino acids for new protein synthesis. Clearance of used intracellular insulin fragments improves insulin-mediated cellular signal transduction mechanisms to stimulate glucose uptake [62,63]. Decreased insulin-degrading enzyme in type 2 diabetic rat models results in significant decrease of insulin catabolism (15%-30% reduction) [64], thus increasing the incidence of type 2 diabetes [65,66]. Zinc is also an integral part of aminopeptidase, which is the insulin-regulated membrane enzyme that cleaves several peptidases in the circulating system and regulates GLUT4 metabolism [22]. Thus, zinc nutrition is extremely important in the prevention and treatment of mammalian diabetes. However, treatment of diabetes with either zinc or CHP alone was minimally effective [15,16]. Therefore, treatment of diabetes is effective only when CHP

plus zinc are combined in a dose-dependent manner [14-16] because of the ability of CHP to stimulate zinc uptake in muscle cells [13].

The current data (Figs. 1-7, Tables 2 and 3) and previous reports [13-16] clearly indicate that energy restriction by RVF diet and physiochemical adjustment to improve insulin sensitivity by HCS synergistically ameliorate insulin resistance in genetically diabetic G-K rats and in obese aged S-D rats. Specifically, beneficial effects of RVF diet are primarily (1) energy restriction to balance energy intake and expenditure resulting in negative energy balance, which reduces BW and incidence of diabetes [67,68]; (2) adjustment of satiety control endocrinal system to reduce food and water intake and improve insulin resistance in diabetic rats because food intake did not decrease in obese rats treated with RVF alone but decreased food intake in G-K rats in later days; and (3) glycemic control because BW was not affected significantly by RVF-alone treatment in both diabetic and obese S-D rats. On the other hand, only the HCS diet treatment affects BW in both diabetic G-K and obese S-D rats, whereas RVF diet alone did not affect BW in both rats. Thus, RVF decreases the rise in blood glucose [4-16,53], and HCS is important in the control of BW because RVF diet alone did not lead to the same benefit as HCS, which contains isoflavones to stimulate insulin sensitivity [11,12] and contains CHP precursors and CHP plus zinc to stimulate glucose utilization [13-16]. However, HCS is capable to control BW, although the exact mechanisms are not clearly understood. The most important role of HCS is to stimulate zinc metabolism in the body because CHP in HCS regulates zinc metabolism in muscle cells [13].

Zinc stimulates insulin receptor β-subunit autophosphorylation [25,26], is an integral part of insulin-degrading enzyme required for the clearance of used insulin fragments and proteins in the cells [41-44], and is an integral part of an enzyme related to the gene expression of GLUT4 [22,69]. Thus, a diet consisting of RVF + SPH contributes to the blood glucose control by energy restriction and improves both insulin resistance and weight loss independent of decreased food consumption.

In conclusion, this study demonstrated evidence that (1) consumption of controlled-energy diet, such as RVF, significantly improves blood glucose control by reducing energy intake; (2) HCS, which contains substantial amounts CHP, further improves impaired insulin resistance based on the fact that HCS improves TAFGC (Fig. 1A) under decreased plasma insulin levels (Fig. 2A) in diabetic G-K rats and improves BW control in both rat groups; and (3) RVF + HCS is the better diet than RVF or HCS alone in the control of blood glucose and BW. Thus, RVF + HCS is potentially an excellent functional food in the control of blood glucose metabolism in genetically diabetic G-K rats and in reducing BW in aged overweight S-D rats (Figs. 1-7) without posing any adverse side effects (Tables 2 and 3).

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