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RESEARCH ARTICLE

Hypocholesterolemic and hypoglycemic effects of enzymatically modified carbohydrates from rice in high-fat-fed C57BL/6J mice

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Scope: Enzymatically modified rice starch (ERS) synthesized with 4-α-glucanotransferase has a longer structure than rice starch, which could delay digestion, similar to dietary fiber. We investigated the effects of ERS on glucose and lipid metabolism with mice fed a high-fat diet containing ERS (HFD-ERS).

Method and results: Four weeks of ERS feeding showed hypoglycemic effects with a significant reduction in fasting glucose (46%), insulin (57%), and leptin (83%) levels; improved glucose tolerance (20% in AUC of oral glucose tolerance test); and increased adiponectin concentrations (+27%) compared to the HFD group. Notably, phosphorylation of AMP kinase (AMPK) was markedly induced in the HFD-ERS livers compared to HFD livers. Additionally, ERS significantly reduced total cholesterol concentrations with induction of fecal bile acid excretion (+21%, $P<0.05$) in the HFD-ERS group compared to the HFD group. The mRNA and protein expressions of hepatic LDL receptors were significantly induced. However, cholesterol 7 alpha-hydroxylase (CYP7A1) expression was downregulated possibly due to induction of intestinal farnesoid X receptor (FXR; +2.4-fold, $p<0.05$) and fibroblast growth factor-15 (FGF-15; +2.2-fold, $p<0.01$).

Conclusion: Our data suggest that ERS feeding may have hypoglycemic and hypocholesterolemic effects via a mechanism similar to that of dietary fiber.

Keywords:
Bile acid excretion / Cholesterol / Glucose metabolism / Lipid metabolism / Rice starch

1 Introduction

The prevalence of hyperlipidemia, the major risk factor for cardiovascular disease, is increasing worldwide [1] and the role of plasma lipids in the etiology of coronary heart disease (CHD) has been well-defined. A high plasma concentration of total cholesterol (TC), triglycerides (TG), and LDL cholesterol and a low plasma concentration of HDL cholesterol are considered important risk factors for CHD [2].

Type 2 diabetes mellitus (T2DM), which is also a major health problem worldwide, can cause hypertriglyceridemia and CHD [3], and its prevalence has been dramatically increased worldwide during last few decades [4]. T2DM is

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Abbreviations: ASBT, apical sodium-dependent bile acid transporter; **AUC,** area under the curve; **CHD,** coronary heart disease; **CYP7A1,** cholesterol 7 α-hydroxylase; **DP,** degree of polymerization; **ERS,** enzymatically modified rice starch; **FGF-15,** fibroblast growth factor-15; **FXR,** farnesoid X receptor; **HFD,** high-fat diet; **HFD-ERS,** high-fat diet containing ERS; **HMG-CoA reductase,** 3-hydroxy-3-methylglutaryl-CoA reductase; **OGTT,** oral glucose tolerance test; **p-AMPK,** phosphorylated AMP kinase; **RC,** rice starch; **TAαGT,** *Thermusaquaticus* 4-α-glucanotransferase; **TBST,** Tris-buffered saline buffer with Tween-20; **TC,** total cholesterol; **TG,** triglycerides; **T2DM,** type 2 diabetes mellitus

characterized by high blood glucose levels with reduced glucose tolerance in the context of insulin resistance, which is associated with the risk for hypertriglyceridemia, atherosclerosis, and obesity [5, 6]. Individuals with T2DM are at a two to fourfold greater risk of developing CHD than healthy individuals [7], and CHD is a major cause of mortality in these patients.

Several effective drugs have been developed to treat hypercholesterolemia and insulin resistance although the risk factors for cardiovascular disease as well as T2DM can be modulated by diet. Based on a meta-analysis, a 1% decrease in plasma cholesterol level can lower the risk of coronary events up to 3%, and this level of cholesterol reduction could be achieved by appropriate food intake such as low-cholesterol and low-fat diets [8], and dietary fiber [9, 10]. In patients who have diabetes with an increased risk of cardiovascular complications, a diet consisting of low fat, high dietary fiber, fruits, and vegetables in foods with low glycemic index is recommended [11].

Dietary fiber intake provides various health benefits, including a reduced risk of developing CHD [12], T2DM [13], and obesity [14]. A meta-analysis revealed that increased dietary fiber consumption improved blood glucose control in T2DM [15] and decreased serum TC [16] although the mechanism by which fiber lowers blood cholesterol remains elusive.

Evidence suggests that some soluble fiber binds bile acids or cholesterol during intraluminal formation of micelles [17]. The resulting reduction in the cholesterol content of liver cells leads to an upregulation of the LDL receptors and thus increased clearance of LDL cholesterol. On the other hand, soluble dietary fiber from cereals has been associated with reduced glucose and insulin responses, apparently due to the retarding effect of soluble fiber on gastric emptying and absorption [18].

Structural modification of carbohydrates could be another important factor for regulating glucose digestion. Generally, starches with higher amylose content are believed to have lower digestion rates, and amylopectin with a high proportion of long chains may moderate starch digestion properties. Based on this hypothesis, Mustapha et al. proposed identifying rice starch (RS) cultivars with a low glycemic response [19]. However, in the previous study, enzymatic synthesis of enzymatically modified rice starch (ERS) possessed increased amylose structure resulting in a disproportional ratio between amylose and amylopectin clusters compared with native waxy starch [20]. These clusters were found less susceptible to pancreatic α-amylase. In fact, the enzyme efficiencies to these amylose and amylopectin clusters were six to eight times lower than those of normal waxy starch. The researchers suggested that this structure may be poorly hydrolyzed by the α-amylase in the small intestine.

In our study, we used ERS enzymatically synthesized from RS with 4-α-glucanotransferase, a disproportionating enzyme. The enzyme breaks the α-1,4-glycosidic bond and transfers a part of the glucan donor molecule to another glucan acceptor by forming a new α-1,4-glycosidic bond. This ERS has increased length on the shorter (degree of polymerization, DP < 8) and longer side chains (DP>24) offering a disproportional ratio between amylose and amylopectin clusters similar to the previous study [21]. Our hypothesis, supported by the previous studies, was that this cluster ratio should have the same slow digestible activity and could mediate glucose and lipid regulation. The actions of modified carbohydrate on glucose and lipid regulation, however, were yet to be identified.

Therefore, in this study, we used ERS supplemented in a high-fat diet (HFD) fed to HFD-induced mild hypoglycemic and hypolipidemic C57BL/6 mice to assess its role in maintaining glucose homeostasis by lowering blood glucose and insulin, and regulating adipokines. We also sought to ascertain its role in lipid homeostasis with blood and hepatic lipid profiles along with cholesterol and bile acid regulation mechanisms.

2 Materials and methods

2.1 Purification of *Thermus aquaticus* 4-α-glucanotransferase

Recombinant *Escherichia coli* containing a thermostable 4-α-glucanotransferase gene isolated from *Thermus aquaticus* was donated by Prof. Kwan-Hwa Park, Seoul National University, Korea. The 4-α-glucanotransferase gene was cloned and expressed in *E. coli*. The recombinant *Thermus aquaticus* 4-α-glucanotransferase (TAαGT) was efficiently purified as described earlier [22] and the purity was electrophoretically confirmed (data not shown). The activity of TAαGT was determined by measuring the optical change in iodine staining during the conversion of amylose by the enzyme [23]. One unit of TAαGT was defined as the amount of enzyme that degraded 0.5 mg/mL of amylose per minute under the assay conditions used. The protein concentration was determined according to the method of Bradford [24] using bovine serum albumin as a standard.

2.2 Preparation of ERS

RS was isolated by the alkaline method [25] from *Ilpumbyeo*, a Korean rice cultivar. Waxy RS was provided by Samyang Genex, Seoul, Korea. A starch suspension (6% w/w) in water was gelatinized in a boiling water bath with stirring for 1 h. The starch paste was then incubated with TAαGT (3 U/g dry starch) at 70°C for 140 h since no significant change in molecular weight profile was obtained. The reaction was terminated by adding threefold volumes of ethanol to precipitate the enzymatically modified starch. The modified starch was obtained by centrifugation (6000 × *g*, 25 min) and purified twice by adding threefold volume of ethanol

following wet-milling and centrifugation. The purified modified starch was dried at 40°C in a drying oven overnight and crushed into a fine powder using mortar and pestle, and passed through a 100-mesh.

2.3 High-performance size exclusion chromatography

Five milligrams of starch sample was completely dissolved in 1 mL of the mobile phase (50 mM $NaNO_3$) to make a 0.5% solution upon boiling for 5 min. The hot sample solution was filtered using a 5.0-μm disposable membrane filter and injected into the high-performance size exclusion chromatography system, which consisted of a solvent delivery module (Prostar 210, Varian, Palo Alto, CA, USA), an injection valve with a 100 μL sample loop (7725i, Rheodyne, Cotati, CA, USA), a differential refractive index detector (Prostar 355, Varian), and two SEC columns in combination (G5000 PW, 7.5 × 600 mm and G3000 PW, 7.8 × 300 mm, Tosoh, Tokyo, Japan). The columns were kept at room temperature. The flow rate of the mobile phase was set at 0.4 mL/min.

2.4 High-performance anion exchange chromatography

A starch sample (6 mg) was dissolved in 0.6 mL of 50 mM sodium acetate buffer (pH 4.5) upon boiling for 15 min with intermittent mixing. The solution was further treated with isoamylase (10 μL, × 10, 158 U/mg, 1000 U/mL, Megazyme, Bray, Ireland) at 40°C for 3 h. The reaction was terminated by boiling for 15 min. The debranched sample solution was filtered through a 0.45-μm membrane filter (Millipore, Billerica, MA, USA). The composition of the starch branched chains was analyzed using a high-performance anion exchange chromatography system. The filtrate (200 μL) was injected and analyzed using a CarboPacTM PA1 column (250 × 4 mm, Dionex, Sunnyvale, CA, USA). The system was equipped with a pulsed amperometric detector (ED40, Dionex). Two eluents, A and B, were 150 mM sodium hydroxide and 150 mM sodium hydroxide in 600 mM sodium acetate solution, respectively. The eluent gradients were operated at a flow rate of 1 mL/min.

2.5 Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy

For further examination of the degree of branching, ^{1}H NMR analyses of starch samples were performed using an AVANCE 600 NMR spectrometer (Bruker, Rheinstetten, Germany) operating at 600 MHz according to the method of Gidley [26]. Native starches were pretreated in DMSO. Starch dispersions (1% w/v) in 90% DMSO were boiled and stirred at 95°C for 2 h, followed by continuous stirring at room temperature for 24 h. The obtained solutions were precipitated with threefold volumes of ethanol and the precipitates were separated by centrifugation at 6000 × *g* for 15 min. The precipitates were purified and dehydrated by adding three-fold volumes of ethanol twice, dried at 40°C, and ground into a fine powder. For ^{1}H NMR measurements, a sample (1.2 mg) was dissolved in 0.6 mL of deuterium oxide (D_2O) and ^{1}H NMR spectra were obtained at 80°C.

2.6 Animals and feeding

Thirty 6-wk-old C57BL/6J male mice were purchased from Samtako Bio Korea (Seoul, Korea). After 1 wk of acclimatization, a feeding protocol was initiated. Mice were randomly assigned into three groups: the control group with normal chow (control, $n = 10$, AIN-93G) feeding, HFD group (HFD, $n = 10$, 60% total calories from fat), and high-fat diet containing ERS (HFD-ERS, $n = 10$, 60% total calorie from fat) group. In the HFD-ERS group, dextrose in 60% HFD was replaced with ERS (data in Supporting Information Table 1). Both HFD and HFD-ERS groups were fed HFDs for 2 months prior to the 4-wk off feeding period. In short, both HFD and HFD-ERS groups were fed HFDs for 2 months, then, the HFD-ERS group was fed HFD-containing ERS for 4 wk, whereas HFD and control groups continuously fed high-fat and control AIN-93G diet for 4 wk. Mice were maintained in a 12-h light–dark cycle. Distilled water was supplied as drinking water ad libitum. Blood samples were collected retro-orbitally at baseline (before background diet), 0 wk (after 2 months of background diet), and 4 wk after test diet feeding with intraperitoneal injection avertin (2-2-2 tribromoethanol, Sigma, St. Louis, MO, USA). Blood samples were collected in the EDTA tube, and plasma was separated after centrifugation at 12 000 rpm for 20 min and stored at −20°C until analysis. Mice were killed after 4 wk of test diet and tissue samples including the liver, intestine, adipose tissue, and muscle were collected, briefly washed with phosphate-buffered saline, and then stored at −80°C until analysis. All animal experiments were performed according to the approved protocol by the Institutional Animal Care and Use Committee of Korea University (Protocol No. KUIACUC-20090420-4).

2.7 Plasma lipid, glucose, oral glucose tolerance test (OGTT), adiponectin, and insulin

Plasma TGs and total, LDL, and HDL cholesterol levels were enzymatically analyzed with a Cobas C111 automatic analyzer (Roche, Basel, Switzerland) using assay kits from Roche. OGTT was performed after 4 wk of test diet feeding following an overnight fasting for 16 h. Bolus of glucose solution (0.25 g/kg body weight) was gavaged, then blood

glucose concentrations were measured at 0, 30, 60, 90, and 120 min with an Accu-Check glucometer (Roche). The plasma insulin and adiponectin levels were quantified with ELISA kits from Alpco (Salem, NH, USA) and Invitrogen (Carlsbad, CA, USA), respectively.

2.8 Fecal bile acid and hepatic lipid measurements

Stool samples were collected during the last 5 days of the test diet feeding, followed by freeze-drying and grinding. Then bile acid was extracted using the method of Fausa and Skålhegg with minor modifications [27]. In brief, 0.2 g stool sample was mixed with 2 mL 100% methanol and held overnight with agitation. Then after centrifugation, the supernatants were concentrated up to 0.5 mL. Total bile acid concentration was analyzed using a total bile acid assay kit (Bio-Quant, San Diego, CA, USA) according to the manufacturer's protocol. Hepatic lipid was measured according to the method by Lihe Zhang et al. [28]. In brief, 40 mg of mouse liver tissue was homogenized with 800 μL HPLC-grade acetone and incubated overnight. Then, 50 μL of acetone-extract lipid suspension was taken to measure hepatic cholesterol with an Amplex Red Cholesterol Assay Kit (Invitrogen) and hepatic TG levels with an enzymatic assay kit using the Cobas C111 according to the manufacturer's instructions. Hepatic lipid content was expressed as milligrams of TG and TCH per gram of liver tissue.

2.9 RNA isolation and quantitative real-time PCR

Total RNA was isolated from the liver and intestine of different mice groups by using an RNAiso Plus Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. For cDNA synthesis, 2 μg of total RNA was reverse-transcribed with oligo dT and PrimerScriptTM Reverse Transcriptase (Takara Bio) according to a protocol creating 20 μL of DNA. The gene-specific mouse primer list is provided in Supporting Information Table 2. All primers were designed by nucleotide blast software from the National Center for Biotechnology Information (NCBI) and purchased from Bionics (Seoul, Korea). Quantitative real-time polymerase chain reaction was performed with the iQ SYBR Green Supermix reagent (Bio-Rad, Hercules, CA, USA) using the iQ5 iCycler system (Bio-Rad). The PCR reactions were conducted at 95°C for 3 min followed by 60 cycles at 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. A melt curve of 71 cycles starting at 55°C and increasing by 0.5°C every 10 s was carried out to determine the primer specificity. Expression levels of each gene were normalized with their corresponding β-actin values and analyzed using iQ5 System Software (version 2) with the normalized expression method according to the manufacturer's guidelines.

2.10 Western blotting

Liver tissues of each mouse were homogenized with a homogenizer (IKA® T10 Basic, Guangzhou, China) in RIPA buffer (1 M Tris-HCl [pH 7.6], 220 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) and protease inhibitor cocktail mixed at a ratio of 100:1. Cell degradation was performed with three consecutive thaw–freeze cycles in liquid nitrogen. The homogenates were then centrifuged at 12 000 rpm for 10 min at 4°C. The protein concentration was determined with the BCA kit (Bio-Rad). Proteins (50 μg) were subjected to SDS-PAGE (10 or 12% gel) and then transferred and immobilized on a nitrocellulose membrane. After blocking with 5% nonfat milk in Tris-buffered saline buffer with Tween-20 (TBST, final concentration 0.1%), the membrane was stained by Ponceau S. After washing with TBST, the membrane was incubated with primary and secondary antibodies with subsequent TBST washing in between two incubation periods. Protein expressions were detected and calculated from the intensity of the bands formed using the Chemidoc XRS+imaging system (Bio-Rad). Different protein expression levels were assayed with the primary and their corresponding secondary antibodies, e.g. α-tubulin (mouse monoclonal IgG and anti-mouse IgG HRP), phosphorylated AMP kinase (p-AMPK) (rabbit polyclonal IgG and anti-rabbit IgG), 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) (goat polyclonal IgG and anti-rabbit IgG), LDL receptor (rabbit polyclonal IgG and anti-rabbit IgG), cholesterol 7 α-hydroxylase (CYP7A1) (rabbit polyclonal IgG and goat anti-rabbit IgG), farnesoid X receptor (FXR) (goat polyclonal IgG and donkey anti-goat IgG-HRP), fibroblast growth factor-15 (FGF-15) (goat polyclonal IgG and donkey anti-goat IgG-HRP), and apical sodium-dependent bile acid transporter (ASBT) (goat polyclonal IgG and donkey anti-goat IgG-HRP). All the primary and secondary antibodies were diluted with TBST at a ratio of 1:2000 to have working solutions. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.11 Statistical analysis

All data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Student's *t*-test was performed for group comparisons. Correlations among different variables were assessed by Pearson's correlation coefficients. Data were deemed to be significantly different at $p < 0.01$ and $p < 0.05$.

3 Results

3.1 Structural characteristics of ERS

The 4-α-glucanotransferase (EC 2.4.1.25) (also called D-enzyme) is involved in starch metabolism. The TAαGT-

modified RS (ERS) exhibited unique molecular structural characteristics compared with those of the native RS upon enzymatic reaction. The molecular weight profile (Fig. 1A) revealed that the average molecular weight of the ERS reached approximately 10^5 Da corresponding to approximately 550 glucose units after enzymatic reaction and did not decrease further after prolonged incubation. Branch chain distribution of ERS (Fig. 1B) indicated that the shorter (DP < 8) and longer α-1,4 glycosidic chains (DP>24) apparently increased, but the intermediate α-1,4 glycosidic chains (DP = 8–24) decreased compared with those of RS. This enzyme action mode (disproportionation of α-1,4 glucans) was confirmed by the change in the ratio of α-1,4 to α-1,6 linkages in ERS (Table 1) using ^{1}H NMR (Fig. 1C). The ratio of α-1,4 to α-1,6 linkages in ERS compared with control RS was unaltered (Table 1).

3.2 Effect on the plasma glucose, insulin, and OGTT

At 0 wk, the HFD and HFD-ERS groups showed significantly increased fasting glucose concentrations by 2.5- and 2.6-fold ($p<0.01$), respectively, compared with controls, and the levels were similar between the HFD and the HFD-ERS groups. However, at 4 wk, fasting glucose levels showed a significant reduction (46.1%, $p<0.01$) in the HFD-ERS group compared with the HFD group (Fig. 2A) and fasting glucose levels in the HFD-ERS group became similar to the level in the control group. A similar trend was observed for plasma insulin levels (Fig. 2B). At 4 wk, the HFD group had 2.7 times higher ($p<0.01$) insulin levels than the control group, whereas insulin levels in the HFD-ERS group were reduced by 57.3% ($p<0.01$) compared with the HFD group. Compared with

Table 1. Relative amount (%) of α-1,4 and α-1,6 linkages in the samples measured using 1H NMR, where peaks at 5.4 and 5.0 ppm were assigned to H-1 of α-1,4 and α-1,6 linkages, respectively

Sample	α-1,4 Linkages	α-1,6 Linkages	α-1,4:α-1,6
Panose	47.7	52.3	~1 (0.91)
RS	96.3	3.7	~26 (26.0)
Waxy RS	93.7	6.3	~15 (14.9)
ERS	96.1	3.9	~25 (24.6)

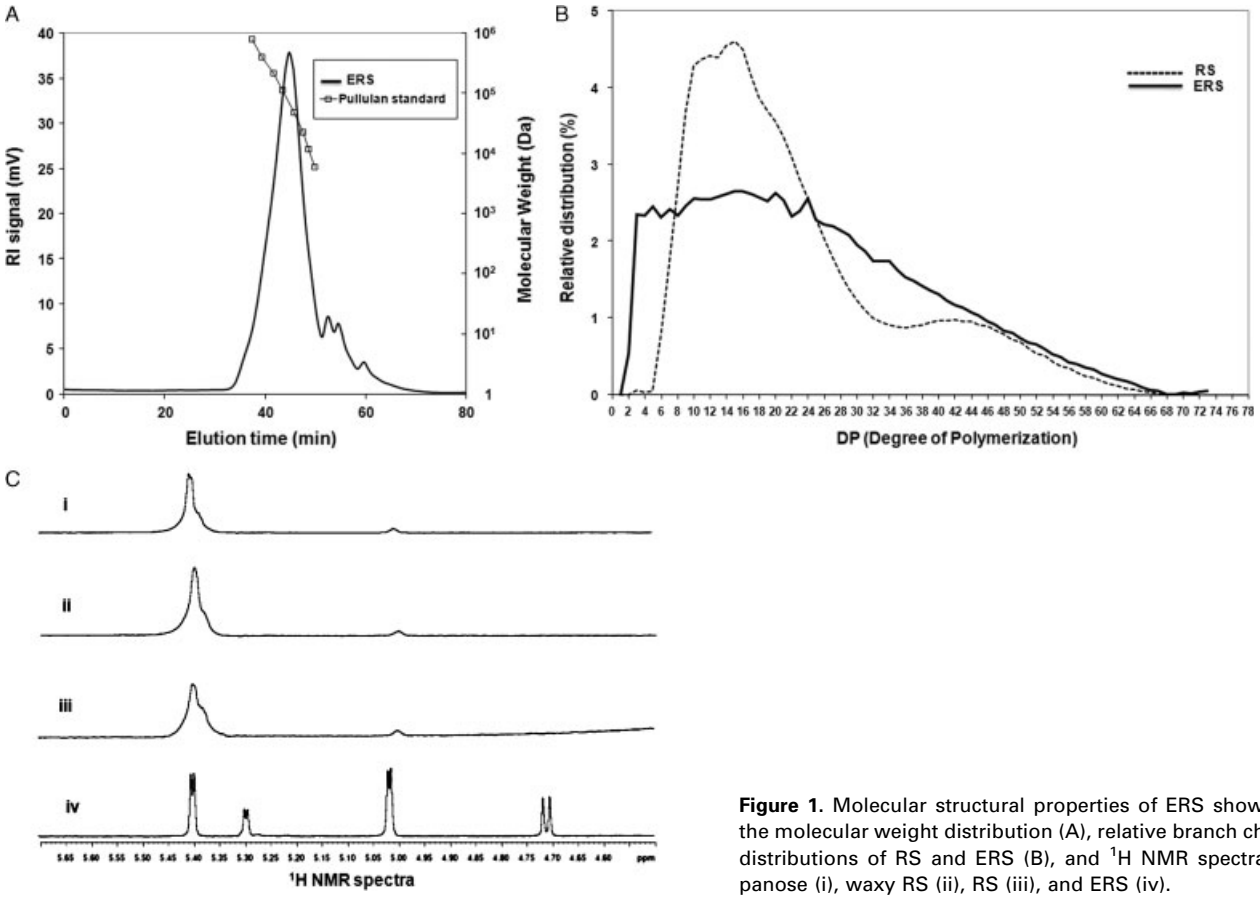

Figure 1. Molecular structural properties of ERS showing the molecular weight distribution (A), relative branch chain distributions of RS and ERS (B), and ^{1}H NMR spectra of panose (i), waxy RS (ii), RS (iii), and ERS (iv).

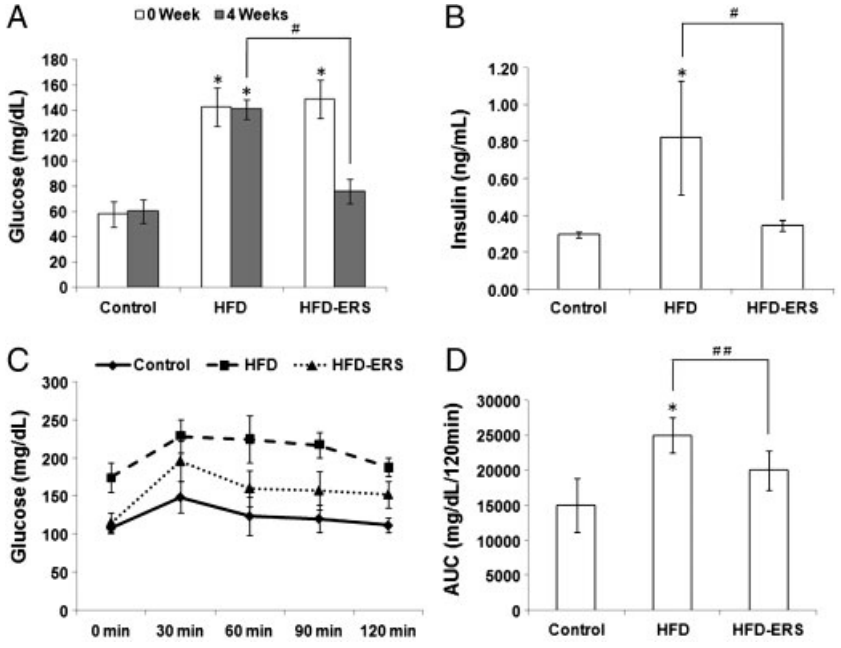

Figure 2. Four-week treatment effects of HFD-ERS on plasma glucose (A), insulin (B), OGTT (C), and AUC of OGTT (D) content in control, HFD, and HFD-ERS groups. Plasma glucose was compared before (0 wk) and after (4 wk) treatment. Other parameters were measured after treatment. For OGTT comparison in different groups, glucose was measured from the tail vein after 2 h of oral administration of D-dextrose at 0.25 g/kg in overnight fasted mice. OGTT of different groups at different timescales and AUC at 120 min were prepared. Data are expressed as mean ± SEM with $n = 10$ ($n = 3$ for OGTT). Asterisk stands for $p < 0.01$ versus control, whereas # and ## represent $p < 0.01$ and $p < 0.05$ versus HFD, respectively.

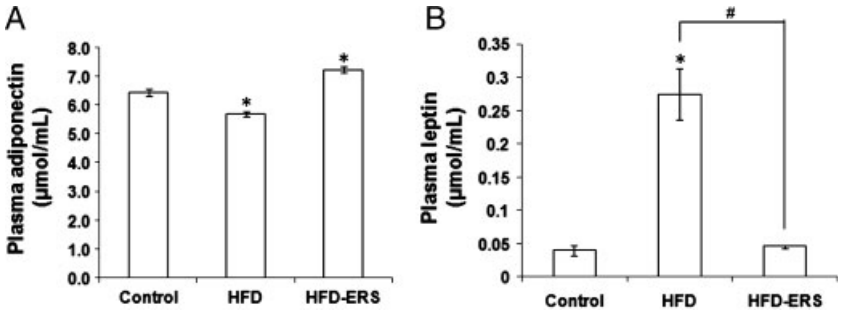

Figure 3. Plasma adipose-derived hormone levels in different groups after a 4-wk treatment of HFD-ERS. Adiponectin (A) and leptin (B) were measured by ELISA quantitative determination method. Data shown as the mean ± SEM with $n = 10$. Asterisk indicates $p < 0.01$ versus control and # denotes $p < 0.01$ versus HFD.

the control mice, the HFD group showed markedly delayed plasma glucose removal. However, ERS feeding significantly improved plasma glucose removal, and thus glucose concentrations from 60 to 120 min were significantly lower than those in the HFD group (Fig. 2C). Area under the curve (AUC) values showed significant differences ($p < 0.01$) in the HFD group with a 66.7% increase over the control group (Fig. 2D). However, AUC in the HFD-ERS group was reduced by 20% ($p < 0.05$) compared with the HFD group.

3.3 Plasma adipokines

Plasma adiponectin levels were significantly reduced by 11.4% ($p < 0.01$) in the HFD group compared with controls, but the level was increased significantly ($p < 0.01$) by 26.8% in the HFD-ERS group compared with the HFD group (Fig. 3A). Plasma leptin showed an opposite trend to adiponectin with a 6.9-fold induction in HFD ($p < 0.01$) compared with controls and an 83.4% reduction in the HFD-ERS group ($p < 0.01$) compared with the HFD group (Fig. 3B).

3.4 Effect of ERS on plasma lipid levels

TC levels in the HFD and HFD-ERS groups were significantly higher than in the control group at 0 wk; however, the levels were reduced by 48.2% ($p < 0.01$) in the HFD-ERS group compared with the HFD group at 4 wk (Fig. 4A). Also, a 10.1% reduction in fasting TG levels was observed in the HFD-ERS group compared with the HFD group although the difference was not significant (Fig. 4B). However, TG levels in the HFD-ERS group were significantly higher ($p < 0.01$) than the levels in the normal chow group. HDL concentration was also significantly ($p < 0.01$) reduced in the HFD-ERS group by 17.6 and 44.5% at 4 wk compared with the normal chow and HFD groups, respectively (Fig. 4C). The TC to HDL cholesterol ratio was significantly decreased by 7.48% in the HFD-ERS group compared with the HFD group ($p < 0.05$, Fig. 4D).

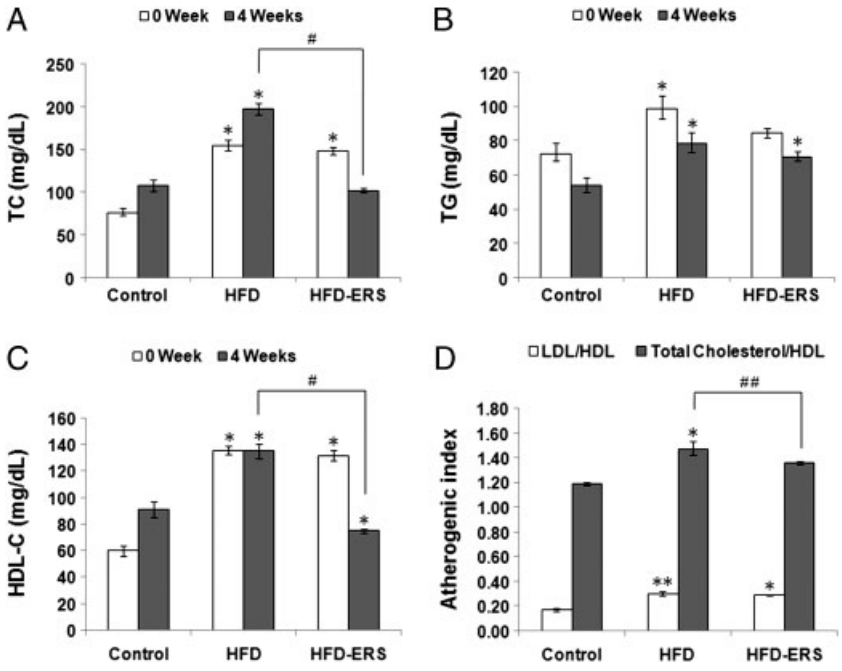

Figure 4. Effect of HFD-ERS after a 4-wk treatment on blood TC (A), TG (B), HDL-cholesterol (C), and atherogenic index (D) of different groups. TC, TG, and HDL cholesterol were compared two times at 0 wk (before treatment) and 4 wk (after treatment). Atherogenic index was prepared from the ratios of TC/HDL and LDL cholesterol/HDL cholesterol data after treatment. All data are expressed as the mean ± SEM with $n = 10$. Asterisks indicate $^{*}p < 0.01$ versus control and $^{**}p < 0.05$ versus control, whereas $^{\#}$ and $^{\#\#}$ denote $p < 0.01$ and $p < 0.05$ versus HFD, respectively.

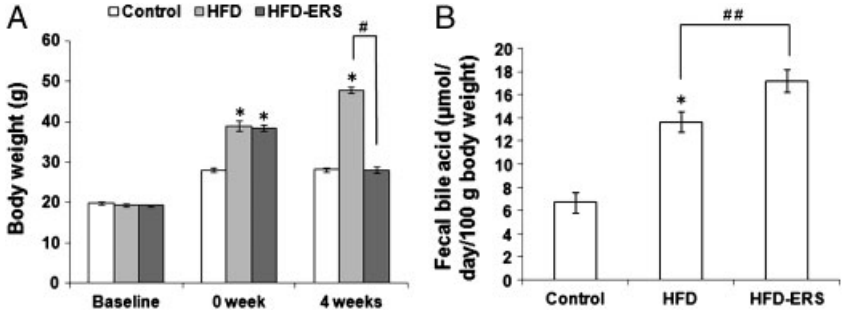

Figure 5. Effect of HFD-ERS on body weight (A) of different groups at baseline (acclimatization), 0 wk (before treatment), and 4 wk (after treatment) and on the fecal bile acid (B) content after 4 wk in different groups. Data are expressed as the mean ± SEM with $n = 10$. Asterisk indicates $p < 0.01$ versus control, $^{\#}$ and $^{\#\#}$ denote $p < 0.01$ and $p < 0.05$ versus HFD, respectively.

3.5 Body weight, fecal bile acid, and hepatic lipid concentrations

At baseline, the body weight of mice was similar among groups (Fig. 5A). However, the HFD for 2 months caused a significant weight gain of 39.4% in the HFD group and 37.5% in the HFD-ERS group at 0 wk compared with controls ($p < 0.01$). After 4 wk of feeding, a dramatic 27.2% reduction in body weight in the HFD-ERS group was observed from that of 0 wk, whereas the HFD group continued to gain body weight, increasing by 22.9% at 4 wk compared with their body weight at 0 wk ($p < 0.01$). In a correlation analysis (Table 2), we found that reduced body weight in the HFD-ERS group was moderately positively correlated with plasma leptin concentration ($p < 0.05$) and mildly positively correlated with plasma TC levels ($p < 0.01$). However, the moderate positive correlation between body weight and adiponectin in the HFD-ERS group was not significant, whereas a significant ($p < 0.01$) slight negative relation prevailed in the HFD group with the same parameter.

Fecal bile acid excretion in the HFD-ERS group was significantly higher by 21.4% compared with the HFD group (Fig. 5B, $p < 0.05$). After 4 wk of feeding, hepatic TG was increased in the HFD group by 2.6-fold ($p < 0.01$) compared with normal chow (Fig. 6A). However, in the HFD-ERS group, the level was reduced significantly by 64.4% compared with the HFD group ($p < 0.01$). Meanwhile, hepatic cholesterol levels were similar among groups (Fig. 6B).

3.6 Cholesterol metabolism gene expression

Key cholesterol gene expression levels were quantified with real-time PCR in the livers and intestines. Compared with the HFD group, the gene expression of hepatic HMG-CoA reductase was significantly ($p < 0.01$) reduced by 79.2% in

the HFD-ERS group after feeding (Fig. 7A), whereas hepatic LDL receptor expression was significantly ($p<0.05$) increased by 57.4% (Fig. 7B). Unexpectedly, a significant ($p<0.01$) reduction by 84.9% was observed in liver CYP7A1 of the HFD-ERS group compared with HFD livers (Fig. 7C). In the intestine, FGF-15 expression was markedly increased (+63.8%, $p<0.01$) in the HFD-ERS group compared with the HFD group, whereas the induction of FXR (+10.3%, $p<0.01$) and ASBT decreased (−37.9%, nonsignificant) (Fig. 7E–G). However, AMPK (Fig. 7D) did not show significant changes among the groups in liver samples.

3.7 Immunoblotting analysis

The expression levels of several key proteins in cholesterol metabolism and phosphorylated AMPK level were measured in livers. No significant difference was observed in the protein levels of HMG-CoA reductase among groups (Fig. 8A). However, the LDL receptor level was significantly increased in the HFD-ERS group compared with the control and HFD groups by 67 and 43.9% ($p<0.01$ and $p<0.05$), respectively (Fig. 8B). CYP7A1 protein expression in the HFD-ERS group was reduced by 28.6% compared with the HFD group (Fig. 8C). In addition, phosphorylated AMPK levels were significantly ($p<0.05$) induced in the HFD-ERS group (Fig. 8D) compared with the HFD group by 71.9%.

The expression levels of the bile acid metabolism proteins were quantified in the intestines (Fig. 8E–G). The HFD-ERS group had a significant increase in FXR by 2.4-fold and FGF-15 by 2.2-fold compared with the levels in the HFD group. ASBT protein levels were reduced by 7.4% compared with the HFD group, but not significantly (Fig. 8G).

Table 2. Correlation of plasma leptin, adiponectin, and TC versus body weights of different groups

	Control	HFD	HFD-ERS
Body weight			
Leptin	−0.436	−0.245	0.661**
Adiponectin	−0.491	−0.058*	0.466
Plasma TC	0.671	0.434	0.093*

Numbers shown are the correlation coefficient (r). Values with asterisks * and ** stand for $p<0.01$ and $p<0.05$, respectively.

4 Discussion

We investigated the hypoglycemic and hypocholesterolemic effects of the ERS diet in HFD-fed C57BL/6J mice to investigate its regulatory mechanisms in the liver and the intestine. Proposed metabolic effects of ERS are summarized in Fig. 9. We found that the ERS diet significantly reduced blood glucose and insulin levels. Enzymatically modified starches have been shown to be poor substrates for digestive enzymes. For example, a study investigated two types of enzymatically modified carbohydrates, one having highly branched amylopectin clusters and the other with highly branched amylase for pancreatic α-amylase kinetics [21]. The studies showed that pancreatic α-amylase possessed higher Km values and very low enzymatic efficiency for both types of modified carbohydrates compared with waxy starch, indicating that modified starch products could be assumed to be poorly hydrolyzed by the digestive enzymes present in the small intestine, thus having a low glycemic index [29]. In our study, branch distributions of shorter and longer side chains (DP<8 and >24) were increased in ERS compared with RS, making its carbohydrate structure less susceptible to α-amylase action in the digestive tract, resulting in delayed absorption of glucose-decreased blood glucose levels.

Prolonged consumption of high glycemic index foods had been implicated in the development of T2DM and prediabetes, cardiovascular disease, and obesity [30], and the intake of low glycemic index foods was shown to be helpful in controlling postprandial glucose levels and preventing the development of diabetes [31]. The reduction in fasting glucose and insulin levels in our study may have been due to low glycemic-like properties of ERS, which thus prevented hyperinsulinemia caused by the overproduction of insulin from pancreatic β cells in an HFD. The OGTT is a well-known clinical test to assess the ability of pancreatic β cells to secrete insulin and the sensitivity of tissues to insulin [32, 33]. We showed that ERS feeding significantly improved glucose tolerance assessed with reduced AUC in OGTT

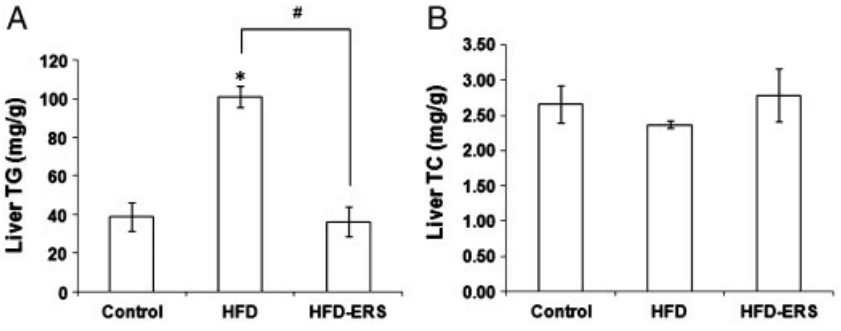

Figure 6. Effect of HFD-ERS on hepatic TG (A) and TC (B) content of different groups after a 4-wk treatment. Data are shown as the mean±SEM with $n=10$. Asterisk indicates $p<0.01$ versus control, whereas # denotes $p<0.01$ versus HFD.

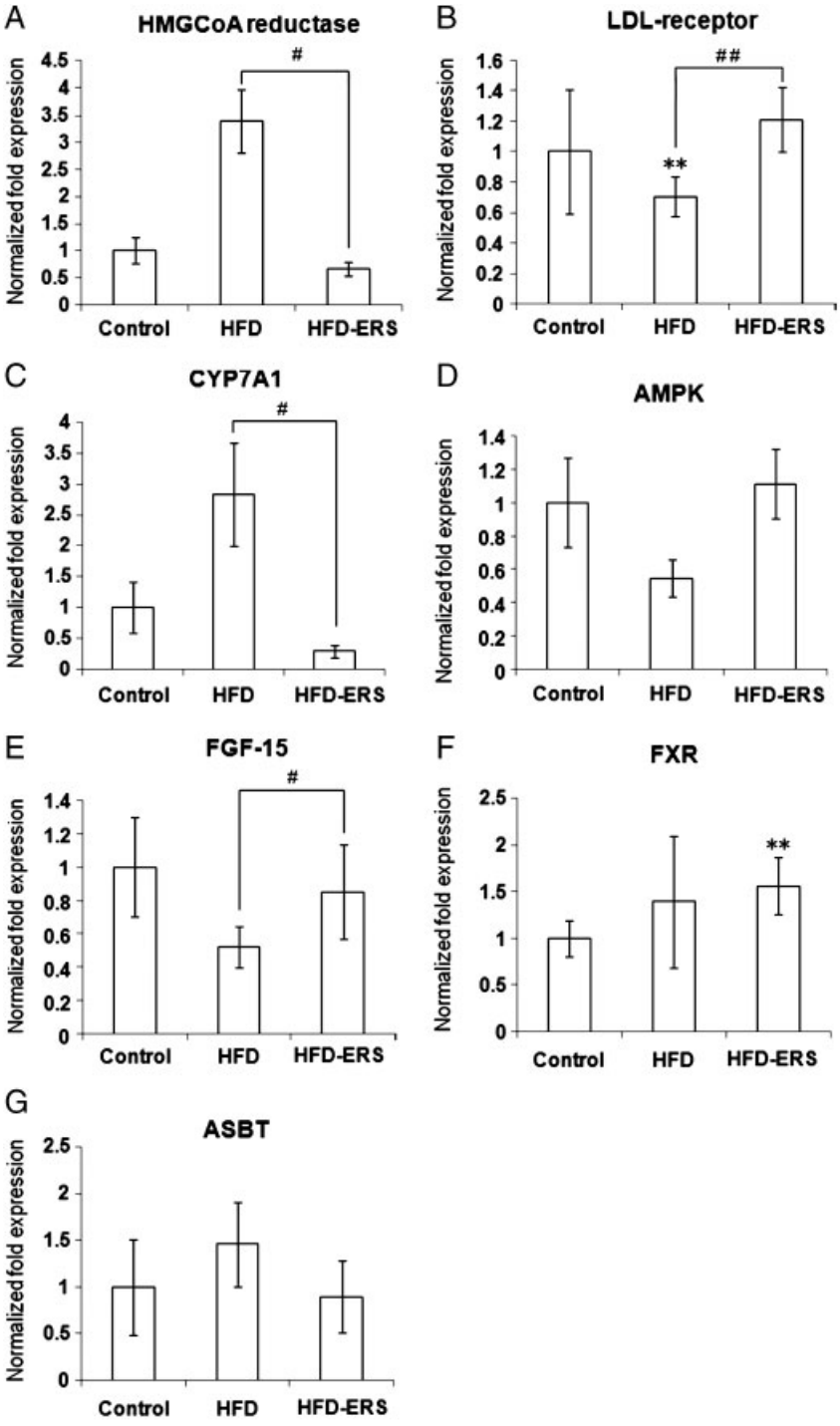

Figure 7. Effect of HFD-ERS on the mRNA expression of lipid and bile acid regulatory genes in the liver (A–D) and intestine (E–G) of different groups by quantitative real-time polymerase chain reaction after treatment. Gene expression levels were normalized with corresponding β-actin values using iQTM5 optical system software from Bio-Rad and graphs were projected based on the variations from the control group. * indicates $p<0.01$ versus control and ** indicates $p<0.05$ versus control; # and ## denote $p<0.01$ and $p<0.05$ versus HFD, respectively.

curves, offering evidence for sound postprandial glucose maintenance in vivo with ERS feeding. It has been shown that carbohydrate-rich diets worsen metabolic parameters for CHD and T2DM with elevating plasma insulin and TG levels and reducing HDL-C concentrations, whereas high fiber diets have hypoglycemic and hypolipidemic benefits with improving plasma glucose, TG levels, and decreasing HDL-C concentrations. The ERS in the present study is a poor substrate for pancreatic α-amylase, and thus may have similar properties to dietary fibers and our data confirmed that the beneficial effects of ERS are similar to those found in high fiber diets.

Improved glycemic condition was assessed using two adipokines levels in response to ERS feeding. Adiponectin

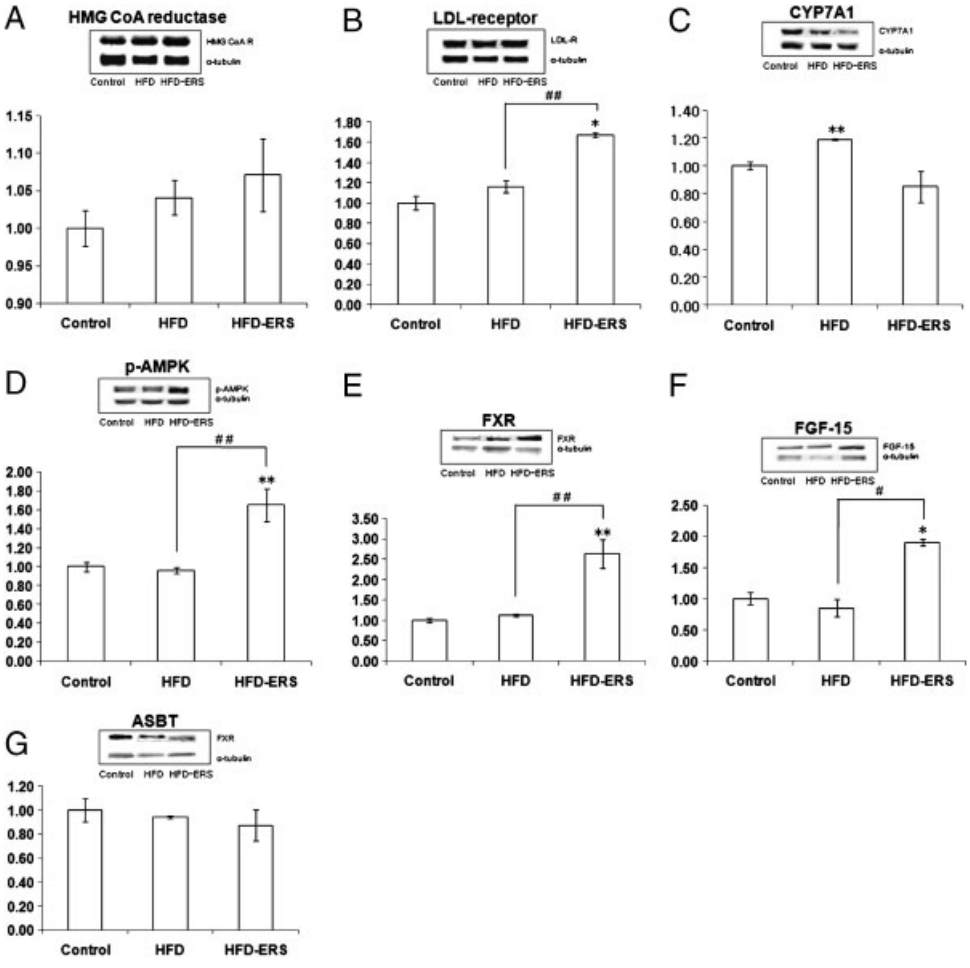

Figure 8. Effect of HFD-ERS on the protein expression of HMG-CoA reductase (A), LDL receptor (B), CYP7A1 (C), and p-AMPK (D) in the liver and FXR (E), FGF-15 (F), and ASBT (G) in the intestine. Data were normalized with α-tubulin and expressed as the mean ± S.E. with $n = 5$. * and ** indicate significant differences from the control at $p < 0.01$ and $p < 0.05$, respectively, and # and ## denote significant differences from the HFD at $p < 0.01$ and $p < 0.05$, respectively.

has an important role in glucose regulation and fatty acid catabolism, whereas leptin is used for energy expenditure in the body. Adiponectin also plays a role in the suppression of metabolic derangements that may result in T2DM [34], obesity, and atherosclerosis [35] and has a protective metabolic and anti-inflammatory function against diabetes [36, 37] although the complete regulation mechanisms have yet to be identified. However, Waki et al. [38] showed that plasma concentration of adiponectin is associated with hepatic AMPK activation, which reduces hepatic glucose production and lipid accumulation, thus preventing insulin resistance in vivo. Our results showed that plasma adiponectin concentration was significantly increased in the HFD-ERS group compared with the HFD group, which may explain, at least in part, improved glucose metabolism after ERS feeding concomitant with phosphorylation of hepatic AMPK. Leptin regulates energy intake and expenditure, including appetite and metabolism [39], and could be helpful for controlling blood glucose and cholesterol level in diabetes. Leptin levels were significantly reduced in the HFD-ERS group compared with the HFD group. Together with adiponectin induction, leptin reduction may contribute to increased insulin sensitivity.

We showed the hypocholesterolemic effects of ERS in mice. TC was significantly reduced and LDL cholesterol concentrations tended to decrease (data not shown), which could provide health benefit against atherosclerosis. Over 80% of cholesterol is carried in HDL in rodents, and thus hypocholesterolemic agents could accompany marginal HDL reduction as well as LDL in mice. In this study, HDL cholesterol levels were slightly reduced in the HFD-ERS group. However, the ratio of TC/HDL, an index [40, 41] for assessing the atherogenic potency of a lipoprotein profile, was significantly lowered in the HFD-ERS group compared with those in the HFD or control groups, indicating that the plasma lipoprotein profile improved significantly after ERS feeding.

Cholesterol gene and protein expression, most notably, mRNA and protein expression of the LDL receptor, was significantly higher in the HFD-ERS group. LDL receptors account for extracellular LDL cholesterol uptake by the liver through a receptor-mediated endocytosis [42]; thus, HFD-ERS increased LDL particle uptake from circulation, and in

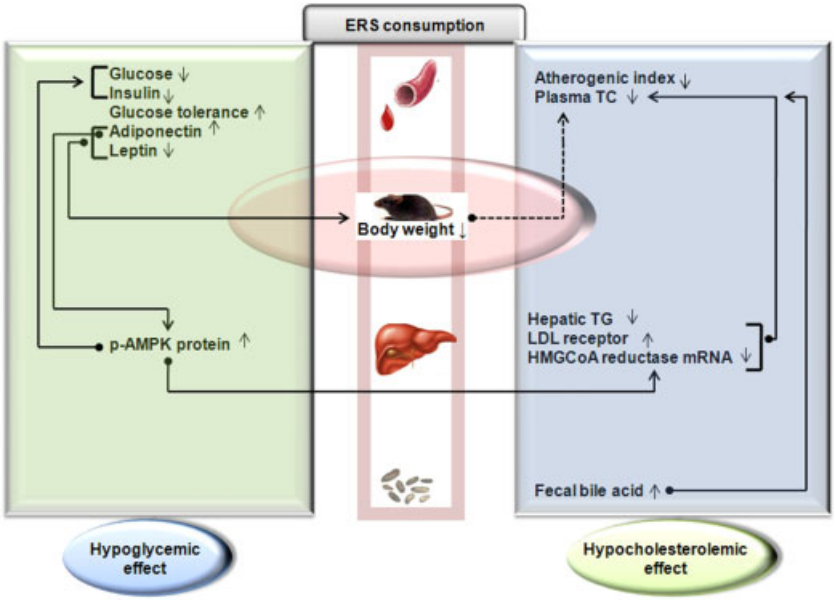

Figure 9. Proposed metabolic pathways for glucose and lipid homeostasis mediated by the HFD-ERS diet in a mild hyperglycemic and hyperlipidemic C57 BL6/J mouse model. Hyperglycemia was controlled by depleting blood glucose, insulin, and leptin, and improving glucose tolerance and adiponectin. However, lipid homeostasis was mediated through reduced plasma TC, atherogenic index, hepatic TG, and mRNA expression of HMG-CoA reductase in the liver. Moreover, elevated fecal bile acid and the induction of LDL-C receptor in the liver were the contributing factors. Hepatic AMPK phosphorylation, stimulated by elevated serum adiponectin, could maintain hyperglycemia by decreasing glucose and insulin and also hypercholesterolemia by reducing HMG-CoA reductase. Body weight reduction was correlated with increased adiponectin and reduced leptin and TC in blood. Increased fecal bile acid, hepatic LDL receptor, and decreased HMG-CoA reductase could be the potential responsible factors for plasma TC depletion.

turn reduced plasma cholesterol concentrations. The HMG-CoA reductase protein was marginally altered but its transcription was markedly induced, which could be explained by increased phosphorylation of AMPK [43]. p-AMPK was shown to inhibit HMG-CoA reductase expression that reduces hepatic cholesterol synthesis. Moreover, p-AMPK itself is stimulated by induction of adiponectin [44] as mentioned earlier; thus, our study implies that ERS feeding results in multiple hypoglycemic and hypocholesterolemic mechanisms interacting among p-AMPK, HMG-CoA reductase, and adiponectin.

Excretion of bile acid, synthesized from hepatic cholesterol, is a major cholesterol removal pathway from the body contributing to cholesterol homeostasis [45]. Our data showed that fecal bile acid excretion was significantly increased in the HFD-ERS group compared with the HFD group after 4 wk of feeding. This may be a major hypocholesterolemic mechanism with ERS.

CYP7A1 in the liver is a gene encoding CYP7A1, a rate-limiting enzyme in the classical pathway for bile acid synthesis [46]. Hepatic cholesterol stimulates CYP7A1 transcription and eventually induces bile acid excretion in the intestine. In our study, HFD-ERS feeding unexpectedly caused a significant reduction in CYP7A1 mRNA and protein expression. Since hepatic cholesterol levels were unaltered, alternate mechanisms should operate the down-regulation. Our results showed upregulation of intestinal FXR and FGF15, negative regulators for CYP7A1 transcription, both in mRNA and in protein levels. FXR, a nuclear receptor for bile acids, suppresses CYP7A1 transcription. FGF-15 secreted in blood binds to hepatic FGF-receptor 4, leading to the repression of CYP7A1 [47, 48].

FXR protein expression was significantly induced in the intestine. Intestinal FGF-15 was induced via a FXR-dependent mechanism, but ASBT protein levels were marginally reduced. ASBT mediates the absorption of secreted bile acids to prevent their loss from the intestine through sodium-dependent electrogenic uptake of bile acid into hepatocytes [49]. However, ASBT levels may not be responsible for increased bile acid excretion.

We also found that ERS did dramatic reduction in the body weight of the HFD-ERS group. Finally, body weight in our study was correlated with TC, plasma leptin, and adiponectin, in accordance with the previous reports [36, 37, 42]. Interpretations of correlation coefficient measurements in this study postulated that reduced body weight could be associated with high plasma leptin and TC and low adiponectin level in accordance with the previous studies [50]. Weight reduction is often induced with elevated plasma levels of adiponectin, which could inhibit the expression of adipogenic genes [51]. Thus, an ERS diet may reduce body weight through blocking the feedback inhibition pathway, resulting in elevated plasma adiponectin levels. Weight reduction is associated with improved parameters in lipid and glucose metabolism [52],

including LDL cholesterol, fasting glucose, and insulin levels. Weight reduction in our study could be mediated by reduced blood glucose, insulin, TC and improved glucose tolerance whose collective performances could offer a sound condition for glucose and lipid homeostasis.

In conclusion, our data suggest that ERS possessed both hypoglycemic and hypolipidemic properties, causing reduced blood glucose, insulin, leptin, and lipid levels, along with liver lipid, and increased adiponectin and fecal bile acid secretion. Elevated LDL receptors in livers favored maintaining lipid homeostasis, and increased FXR and FGF-15 ameliorated fecal bile acid secretion. Thus, it could be a potential functional food offering therapeutic approaches for diabetes and cardiovascular diseases.

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The authors have declared no conflict of interest.

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