ORIGINAL

Treatment with buckwheat bran extract prevents the elevation of serum triglyceride levels and fatty liver in KK-A^{y} mice.

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Abstract : Buckwheat powder or protein has been shown to decrease the total serum cholesterol level in non-diabetic mice or rats. However, the lipid-lowering effect of buckwheat bran extract (BBE) in diabetic mice has not been fully elucidated. KK-A^{y} mice that received six-week treatment with BBE showed decreased body weight and liver weight compared to those of control (vehicle) mice. However, there was no significant difference in food intake. BBE treatments prevented liver triglyceride accumulation and decreased the serum level of triglycerides. In addition, mRNA expression levels lipogenic enzyme genes, fatty acid synthase, acetyl-coenzyme a oxidase and stearyl-coenzyme a desaturase 1, but not those of β-oxidized enzyme genes, were decreased in BBE-treated mice. Level of transcription factors ChREBP and SREBP1c, transcripts of lipogenic genes, were also decreased in BBE-treated mice. These results suggest that chronic treatment with BBE derivatives could have beneficial effects on hypertriglycemia in patients with type 2 diabetes mellitus. J. Med. Invest. 61 : 345-352, August, 2014

Keywords **:** *buckwheat, triglyceride, fatty liver, lypogenesis*

INTRODUCTION

Type 2 diabetes mellitus is characterized by a chronic hyperglycemic state due to decreased insulin sensitivity in target tissues, including skeletal muscle, adipocytes and the liver, and/or impairment of insulin secretion (1, 2). Obesity is a robustly pandemic and pathological disease and is responsible for type 2 diabetes mellitus, hyperlipidemia and hypertension (3). Increased serum levels of free fatty acid (FFA) or triglyceride (TG) deteriorate hyperglycemia through peripheral insulin resistance, finally

Abbreviations used
PBS : phosphate buffered saline, GPO : glycerol-phosphate oxidase, DAOS : sodium n-ethyl-n-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxy aniline, ACS : acyl-coenzyme a synthetase, ACOD : acyl-coenzyme a oxidase, POD : peroxiase, ACC1 or 2 : acetyl-coenzyme a carboxylase 1 or 2, SCD1 : stearyl-coenzyme a desaturase 1, FASN : fatty acid synthase, ACO : acetyl-coenzyme a oxidase, CPT-1α : carnitine palmitoyltransferase 1a, PPARα : peroxisome proliferator-activated receptor α, TNFα : tumor necrosis factor α, MCP1 : monocyte chemotactic protein 1, ChREBP : carbohydrate responsive element-binding protein, SREBP1c : sterol regulatory element-binding protein 1c.

Received for publication January 20, 2014 ; accepted March 3, 2014.

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resulting in cerebral infarction and cardiovascular disease (4, 5). Thus, in obese type 2 diabetes patients, treatment of hyperlipidemia is clinically important to prevent these comorbidities.

Buckwheat powder is used mainly for making noodles in Asia, while much of buckwheat bran is discarded. Recently, buckwheat has been developed as a functional food for hypertension (6, 7), obesity (7-9). The concentrated buckwheat bran extract (BBE) reduced plasma glucose in streptozotocin-induced type 1 diabetes rats (10) and KK-A^y type 2 diabetes mice (11). As for hyperlipidemia, *in vivo* studies have been suggested that buckwheat powder and protein decrease the level of serum cholesterol or/and serum TG and enhance the exertion of fecal neutral or acid sterol and bile acid (9, 12-16). Insoluble fraction of buckwheat protein associates with cholesterol and reduces micelle cholesterol uptake in caco-2 cells (17). Recently, tartary BBE reduce the serum level of total cholesterol and TG in hyperlipidemic rats (18). However, all *in vivo* studies do not examine the effect of lipid profile in diabetic insulin-resistant animals.

In this study, we investigated the effect of common BBE on obese and diabetic KK-A^y mice and found that BBE treatment decreased serum levels of TG and fatty liver.

MATERIALS AND METHODS

Reagents

Dimethyl sulfoxide (DMSO) was purchased from Nakalai Tesque (Kyoto, Japan). Glucose and insulin were purchased from WAKO (Osaka, Japan). All other chemicals were of analytical grade.

Preparation of extracts from buckwheat bran powder

Common buckwheat (*Fagopyrum esculentum Moench*) or buckwheat bran powder was manufactured by a milling machine at TANI FOOD Co., Ltd. (Ishii, Tokushima, Japan). Ten grams of buckwheat bran powder was added to 50 ml of 80% methanol (v/v) followed by stirring at room temperature for one hour. And then the extracted solution was filtered through filter paper (No. 5B, ADVANTEC, Tokyo, Japan), and the filtrate was concentrated and lyophilized at -80°C. The extracts were resolved with 0.1% DMSO/PBS by sonication (BIORUPTOR Cosmo Bio, Tokyo, Japan) and used for animal experiments. The chemical composition of buckwheat bran powder (moisture ; 4 g, protein ; 2.2 g, lipid ; 0.2 g, dietary fiber ; 89.5 g, sugar ; 2.4 g, ash ; 1.7 g, rutin ; 0.76, quercetin ; 0.001) before methanol extraction was determined according to the method of AOAC (19), and dietary fiber compositions were analyzed according to the method of Prosky *et al.* (20).

Animals

Six-week-old KK-A^y mice (n=12) (CLEA Japan, Tokyo, Japan) were randomly assigned to BBE treatment groups (n=6) and control treatment groups (n=6), respectively. Mice were maintained under specific pathogen-free conditions with a 12-h light : dark cycle at 25 ± 2°C and 55 ± 10% relative humidity. The mice were given a normal chow diet (Oriental Yeast, Tokyo, Japan) and water *ad libitum*. Mice were orally given 1 mg per 20 g body weight BBE resolved with 100 µl 0.1% DMSO/PBS solution or 0.1% DMSO/PBS per 20 g body weight as a control at 10 : 00 AM once per day for 6 weeks. Food intake and body weight were measured and blood samples after overnight starvation were collected from the tip of the tail vein weekly at 10 : 00. Blood samples were immediately centrifuged to collect serum supernatant. Serum samples were stored at -80°C until use for measurement of metabolic parameters. Mice with overnight starvation were sacrificed 6 weeks later under ether anesthesia to obtain tissue samples of the liver, soleus muscle and epididymal fat. The tissues were immediately frozen in liquid nitrogen and stored at -80°C until used for RNA preparation. The tissues were also fixed immediately with 3.8% formaldehyde in phosphate buffered solution (37.5 mM NaH_2PO_4, 183 mM Na_2HPO_4) and stained with H&E stain. All studies were performed in accordance with the ethical guidelines for animal experimentation of the Institution of Health Bioscience, the University of Tokushima and were approved by the institutional review board of the animal ethics committee.

Oral glucose tolerance test (OGTT)

Six-weeks treated KK-A^y mice after sixteen hours starvation were orally administered one gram of glucose per kg of body weight. Blood samples were collected from the tip of the tail vein at 0, 15, 30, 60 and 120 min. Whole blood glucose levels were measured by the FAD-glucose dehydrogenase method with a GLUCOCARD GT-1820 device (ARKLAY, Tokyo, Japan). The average of three measurements at each time for each mouse was used for analysis.

Measurement of lipid parameters

Plasma TG, FFA and total cholesterol concentrations were measured by the GPO-DAOS method, ACS-ACOD method and POD-DAOS method (Wako Pure Chemical Industries, Osaka, Japan), respectively. Lipid in the liver was extracted by the *Folch* method (21), and TG in the liver was measured as described above.

Quantitative real-time RT-PCR

Total RNA was extracted from the liver by using an RNAiso (Takara, Kyoto, Japan) and then total RNAs were reverse-transcribed using a Takara PrimeScript RT reagent kit (Takara, Kyoto, Japan). Quantitative real-time PCR was performed with the LightCycler system (Roche Diagnostics, Switzerland) using Takara SYBR Premix Ex Taq II (Takara, Kyoto, Japan). The following gene-specific primers were used : ACC1 (sense : 5'-aacatccccacgctaaacag-3' ; antisense : 5'-aggtccggaaagagaccatt-3'), ACC2 (sense : 5'-tggagtccatcttcctgtcc-3' ; antisense : 5'-ggacgccatacagacaacct-3'), SCD1 (sense : 5'-gcgatacactctggtgctca-3' ; antisense : 5'-cccagggaaaccaggatatt-3'), FASN (sense : 5'-cccttgatgaagagggatca-3' ; antisense : 5'-actccacaggtgggaacaag-3'), ChREBP (sense : 5'- cctcacttcactgtgcctca-3' ; antisense : 5'-acaggggttgttgtctctgg-3'), SREBP1c (sense : 5'-tacttcttgtggcccgtacc-3' ; antisense : 5'-tcaggtcatgttggaaacca-3') and 18S ribosomal RNA (sense : 5'-aaacggctaccacatccaag-3' ; antisense : 5'-ggcctcgaaagagtcctgta-3'). After the PCR reaction, each PCR product was confirmed for its single amplification by analyzing a melting curve of the PCR products.

Statistical analysis

Data are expressed as means ± SEM. Data were analyzed by the *unpaired Student's t-test*. A p-value < *0.05* was accepted as statistically significant.

RESULTS

Treatment of BBE lowered serum level of TG and fatty liver in KK-A^y mice

To determine the effect of BBE on KK-A^y mice, we decided to use 1 mg BBE/20 g mice weights treatment once a day, which dose is proved recently by us to lower postprandial glucose (22). Body weight in the BBE treatment group (30.37 ± 1.76 g) was significantly decreased compared to that in the control group (36.04 ± 5.05 g) after 6-week treatment (p=0.033). The gain of body weight in the BBE treatment group (5.29 ± 1.76 g) was significantly decreased compared to that in the control group (11.07 ± 3.72 g) after 6-week treatment (p=0.004). Total food intake in the BBE treatment group (160.6 ± 9.7 g) and that in the control group (177.9 ± 21.2 g) were not significantly different (p=0.85).

To determine the effect to insulin-resistance state, we firstly examined OGTT. As shown in Figure 1, the level of blood glucose at 120 min after glucose loading in the BBE group was significantly decreased. However, area under the curve of OGTT, insulin tolerance test, or fasting blood glucose was not changed in the BBE treatment group and that in the control group (data not shown). Next, we

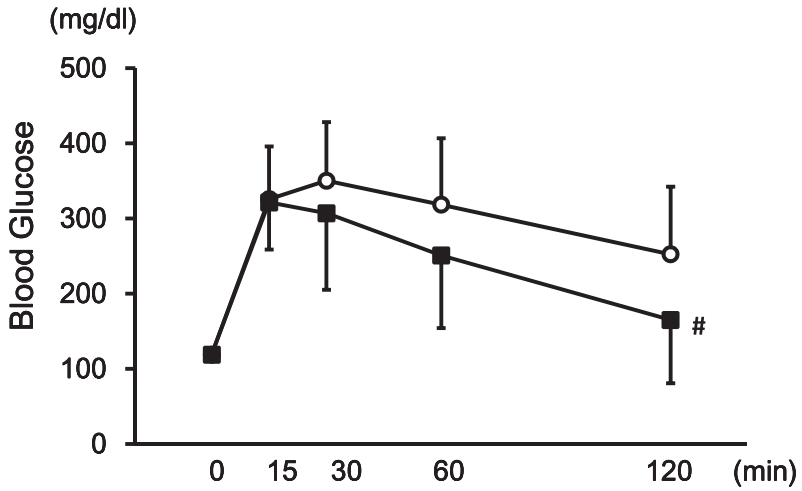

Figure 1. Oral glucose tolerance test in BBE treatment or control treatment groups.
Glucose was orally administered to twelve-weeks-old KK-A^y mice in the BBE group (black square) or control group (white circle) and blood glucose levels were measured at 0, 15, 30, 60 and 120 min. Values are means ± SEM (n=6). $^{\#}: p < 0.05$.

checked the levels of serum total cholesterol, TG and FFA. Only the serum TG levels in the BBE group were significantly lower than those in control group (Figure 2). After sacrificed, the weights of liver but not epididymal fat in the BBE group were significantly lighter than those in the control group (Figure 3). In addition, deposits in the liver in the BBE group had almost disappeared (Figure 4A) and TG content in the BBE group was significantly lower than that in the control group (Figure 4B). Size of fat cells and infiltration of mononuclear cells in the BBE group were not different from those in the control group (Figure 4).

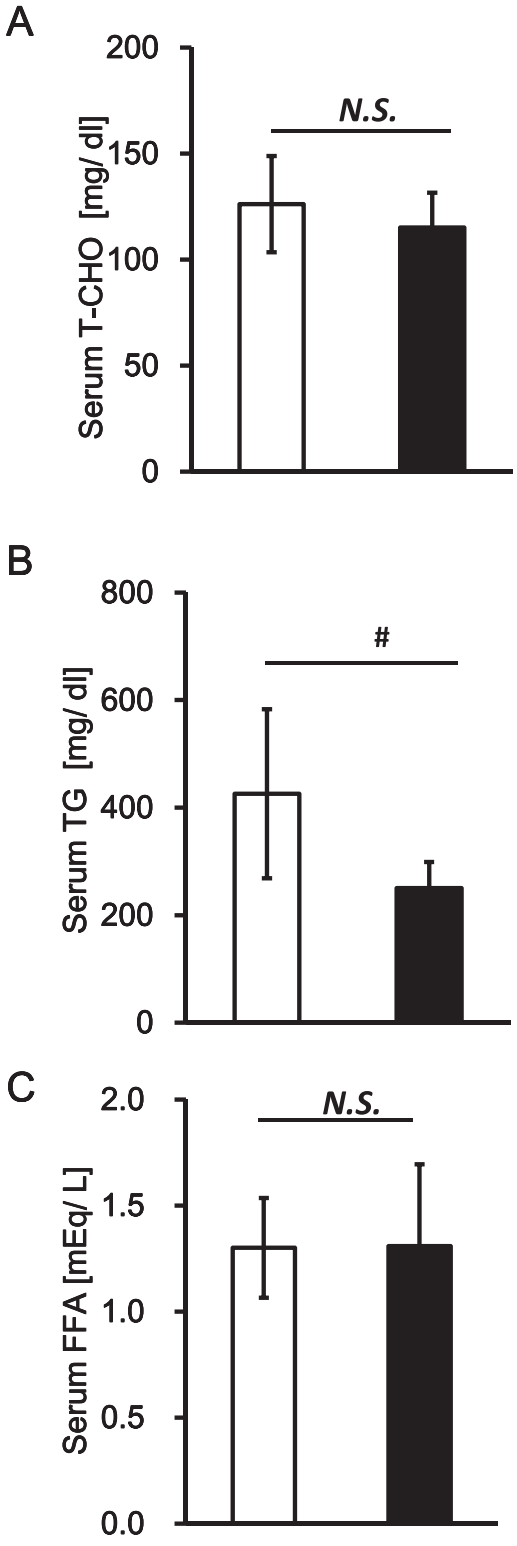

Figure 2. The serum levels of total cholesterol, TG and FFA.
Fasting serum level of total cholesterol (T-CHO), TG and FFA of 12-weeks-old KKA^y mice with BBE (black column) or control treatment (white column) was examined. Data are means ± SEM (n=6). *# : $p < 0.05$.*

The mRNA expression of lipogenic genes was decreased in BBE-treated KK-A^y mice

To evaluate the decreased fat droplets of liver in the BBE group, we examined the mRNA expression of gene related to lipolysis and β-oxidation. The mRNA expressions of lypogenic genes, ACC2, SCD1 and FASN in BBE groups were significantly

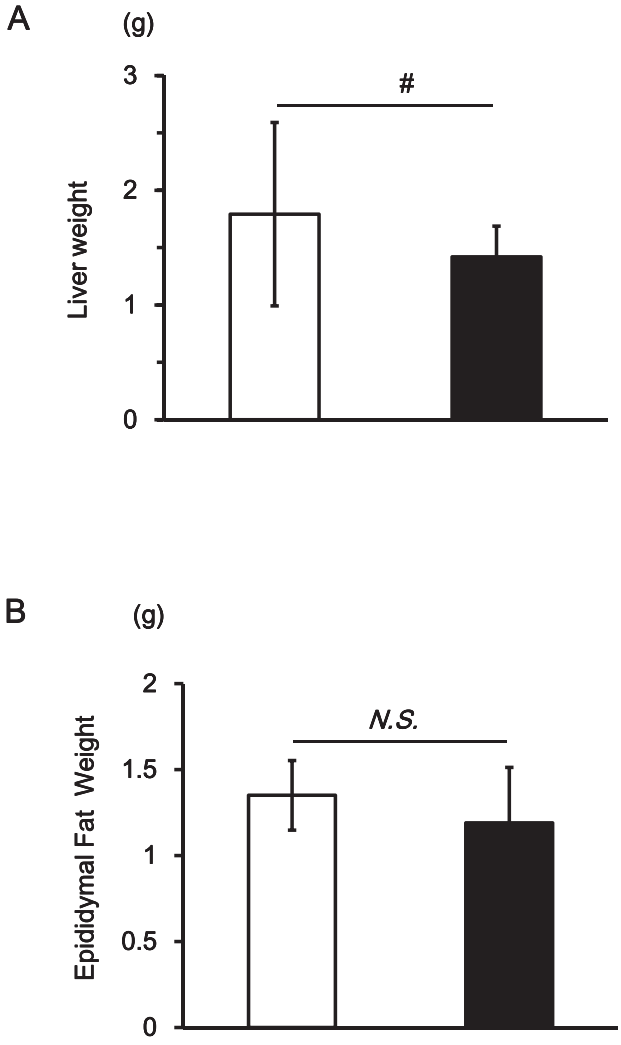

Figure 3. Liver weight in the BBE group was decreased compared to that in the control group.
Twelve-week-old mice in the BBE group (black column) and control group (white column) were sacrificed and liver weights (A) and amounts of epididymal fat (B) were examined. Values are means ± SEM (n=6). *# : $p < 0.05$. N.S. : no significant difference.*

decreased compared to those in the control group (Figure 5). The mRNA expressions of ACC1 in the BBE group also tend to be decreased compared to those in the control group (p=0.063) (Figure 5). Moreover the mRNA expression of SREBP1c transcription factor, which play a central role to lypogenesis in liver, was significantly decreased in the BBE group (Figure 5). The mRNA expression of ChREBP, which is activated by glucose and transcripts lipogenic gene in liver, tended to be decreased in the BBE group (p=0.062) (Figure 5). On the other hand, the mRNA expressions of proteins involved in β-oxidation in liver, such as ACO, CPT1α and PPARα in the BBE group were not different to those in the control group (data not shown).

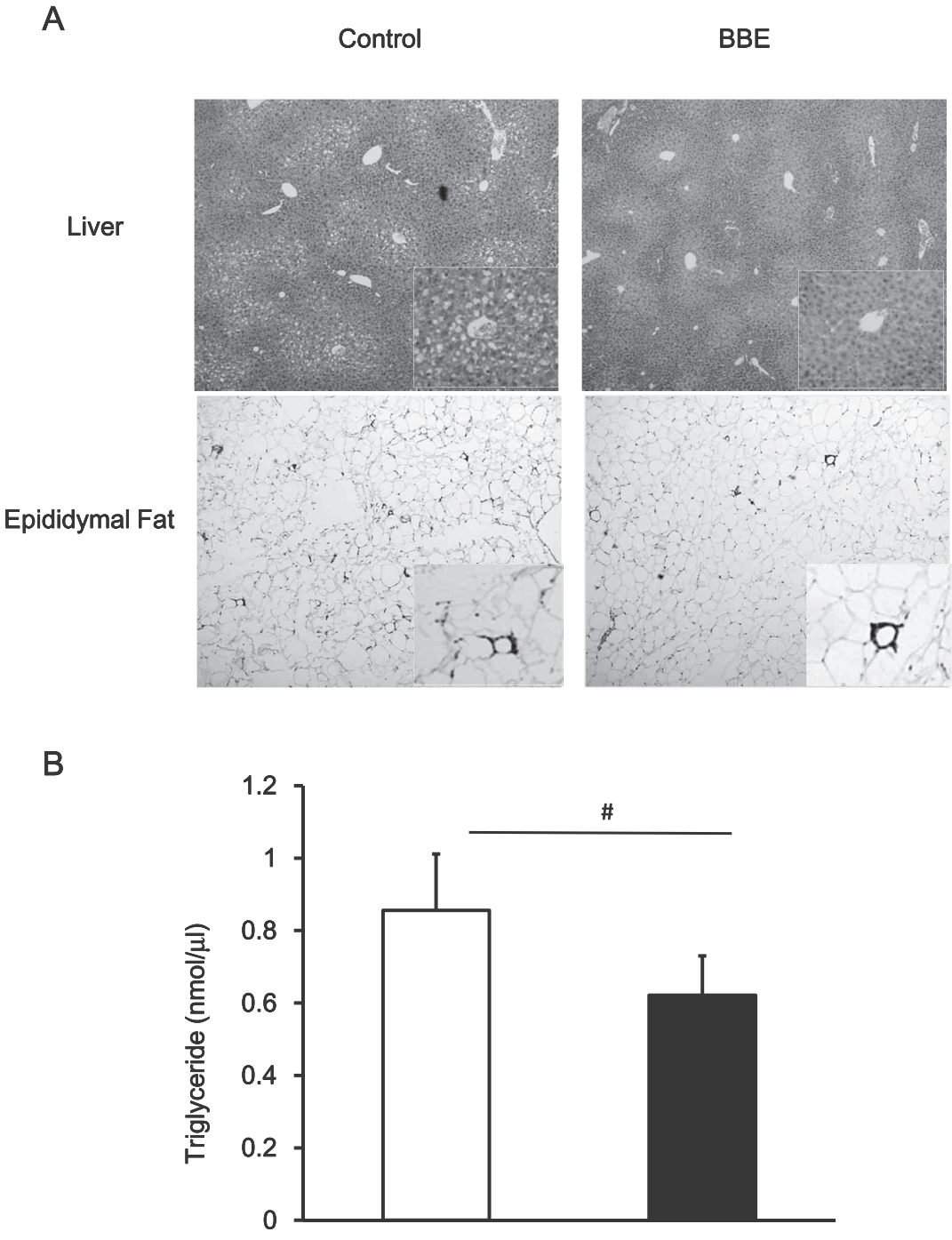

Figure 4. BBE prevents development of fatty liver and TG accumulation.
Twelve-week-old mice in the BBE group and the control group were sacrificed. Livers and epididymal fat were stained with H&E stain (A). Representative images from six mice in each group are presented. TG contents in livers in the BBE group and control group were examined (B) ($^{\#}: p < 0.05$). Values are means±SEM (n=6).

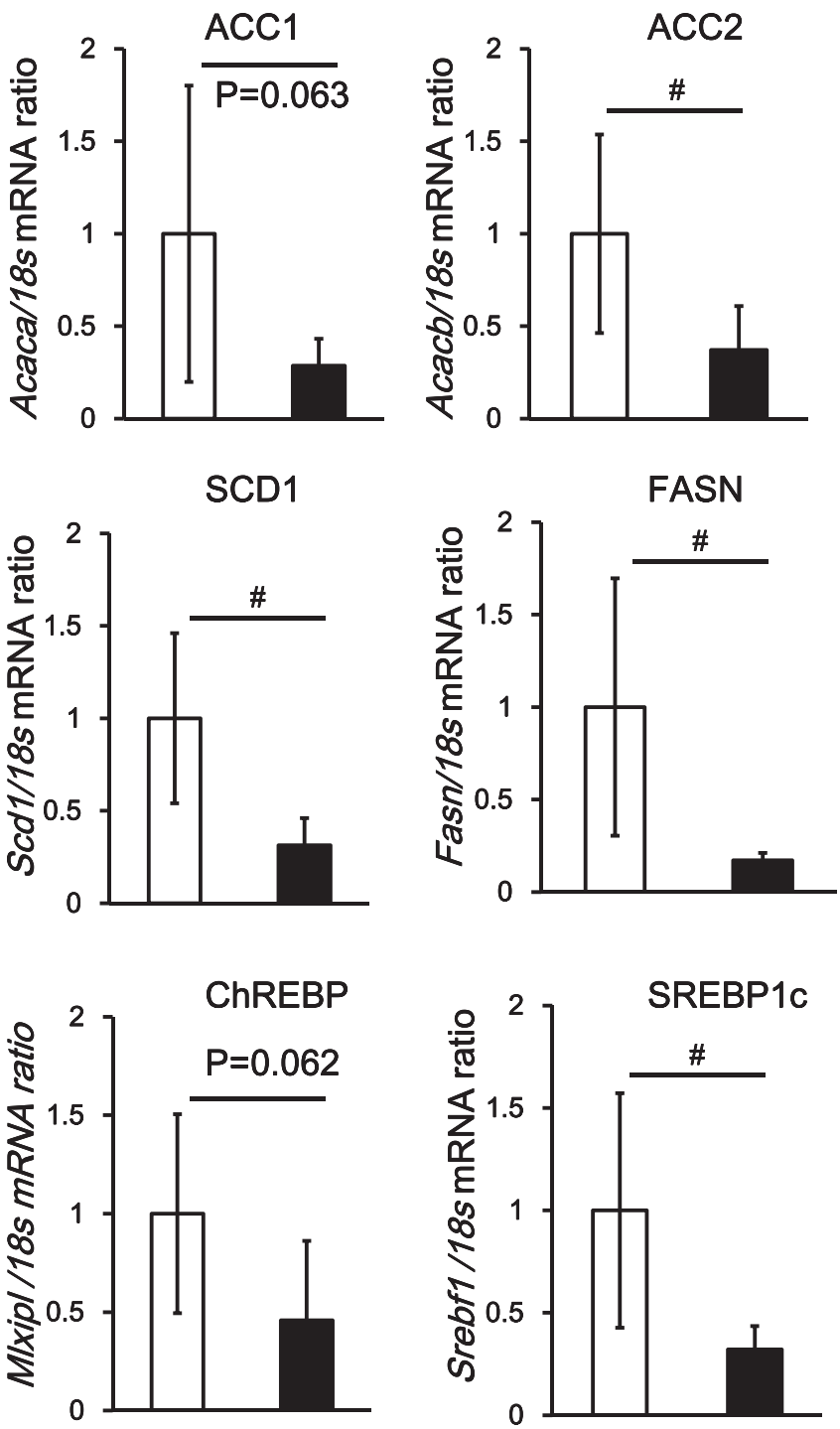

Figure 5. mRNA expression of factors involved in liver lipogenesis.
The liver was obtained from 12-weeks-old KKA^y mice in BBE groups (black column) or control groups (white column). Total RNA was isolated from liver and was subjected to quantitative real-time RT-PCR with primers specific for ACC1/ 2, SCD1, FASN, ChREBP or SREBP1c as described in the *Materials and Methods* section. Data were normalized by 18S ribosomal RNA (*# : $p < 0.05$*). Data are means ± SEM (n=6).

DISCUSSION

In previous studies, buckwheat powder and protein decrease the serum levels of total cholesterol or/and TG in normal rat or rat fed high-fat diet (9, 12-16). In this study, KK-A^y mice treated with BBE showed decreased in body weight, level of TG and fatty liver (Figures 2B, 3A and 4). Moreover, the mRNA expression of lipogenic genes decreased in the BBE group (Figure 5). The water soluble fibers lower the absorption of sterols at intestine that attributes to decrease the level of serum cholesterol (23). The previous studies demonstrated that buckwheat powder and protein exert sterols and/or bile

acid in feces (9, 12-16). It was not reported that insoluble fibers has direct interaction with cholesterol metabolism (24). The fibers of our BBE were almost insoluble fibers, cellulose or hemi-cellulose, which are eluated from methanol. In addition, tartary BBE treatment does not decrease the absorption of cholesterol and increase the cholesterol exertion to feces (18). So we speculated that the effect of BBE on the reducing serum level of TG, but not total cholesterol (Figures 2A, B) was attributed to diminish the lipogenesis on hepatocyte (Figures 4, 5).

Mice in the BBE treatment group showed decreased serum levels of TG (Figure 2B) and recovery of fatty liver (Figure 4). Those results were explained by the decrease of the mRNA expression of lipogenic gene (Figure 5) and were as similar effects as the study of buckwheat protein treatment in lipogenic rat (12). Excess of glucose enters into the liver and metabolizes to acetyl-CoA. The acetyl-CoA finally is used to TG synthesis *via* ACC and FASN cascades (25). Along with palmitate, stearate is catalyzed by SCD1 and finally metabolized to TG (26). Insulin activates SREBP1c and glucose activates ChREBP. Both transcription factors are transcripts of lipogenic genes such as ACC and FASN (24). KK-A^y mice with BBE treatments decreased both of SREBP1c and ChREBP (Figure 5). The CPT-1α is a rate-limiting enzyme for β-oxidation in the liver. The transcription factors PPARα controls lipid metabolism (27, 28). Although we examined the mRNA expression of CPT-1α and PPARα, their expressions was not different in the BBE group and in the control group (data not shown). Those results suggested that BBE treatment influenced lipogenesis, but not β-oxidation in liver.

The previous report suggests that rutin treatment (5 mg/kg body weight) reduces serum levels of cholesterol (29). Oral administration of rutin to streptozotocin-diabetic rats significantly decreases the plasma concentration of total cholesterol and TG (30). The amounts of rutin which they use are much higher than those of our BBE treatment (14 μg per 1 mg BBE) (22). As already described, tartary BBE treatment decreases the serum levels of TG and total cholesterol, and also decreases the serum and liver antioxidants (18). They speculate that increased antioxidant activity after their BBE treatment is derived from rutin. They analyzed the rutin concentration of their BBE. Our amounts of rutin in BBE were near those in their paper. It is suggested that the suppression of oxidative stress decreases pro-inflammation of liver and decreases serum level of TG (31). We checked the mRNA expression of pro-inflammatory adipokines, TNFα and MCP1, in the liver. Both of them were not changed in the BBE group and in the control group (data not shown). From this result, we speculated that the effect of BBE to decrease the serum levels of TG was not be related to decrease the inflammation of liver.

As for the effect of BBE to insulin-resistance, the level of blood glucose at 120 min after OGTT in the BBE group was only significantly decreased compared to that in the control group (Figure 1). Increased serum level of FFA has been known to induce insulin resistance. It is suggested that hypertriglycemia and liver steatosis also induce insulin resistance (32). We found that 1 mg of BBE has inhibitory activities of α-glucosidase (22). We speculated that dose modification of BBE or combination of BBE with anti-diabetic drugs might be more effective for insulin-resistance and hyperglycemia in KK-A^y mice. The decrease of body weight gain in the BBE group could be explained in part the decrease of liver weight. The weight of epididymal adipocyte was not changed in the BBE group and the control group. Therefore, we need to check the effect of BBE to energy expenditures.

Taken together, the results indicated that chronic treatment with BBE derivatives could have a beneficial effect on hypertriglycemia in obese diabetic animals. We are now investigating which phenolic or non-phenolic component(s) of BBE, other than rutin and quercetin, has an effect on TG metabolism.

FOOT NOTE

First three authors contribute equally.

CONFLICT OF INTEREST

The authors declare they have no conflict of interest

ACKNOWLEDGEMENTS

This work was supported by the Local Innovative Cluster Initiative and by a Grant-in-aid for Scientific Research 25350126 (to T.S.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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