Research Article

D-Allulose supplementation normalized the body weight and fat-pad mass in diet-induced obese mice via the regulation of lipid metabolism under isocaloric fed condition

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Scope: A number of findings suggest that zero-calorie D-allulose, also known as D-psicose, has beneficial effects on obesity-related metabolic disturbances. However, it is unclear whether D-allulose can normalize the metabolic status of diet-induced obesity without having an impact on the energy density. We investigated whether 5% D-allulose supplementation in a high fat diet(HFD) could normalize body fat in a diet-induced obesity animal model under isocaloric pair-fed conditions.
Methods and results: Mice were fed an HFD with or without various sugar substitutes (D-glucose, D-fructose, erytritol, or D-allulose, $n = 10$ per group) for 16 wk. Body weight and fat-pad mass in the D-allulose group were dramatically lowered to that of the normal group with a simultaneous decrease in plasma leptin and resistin concentrations. D-allulose lowered plasma and hepatic lipids while elevating fecal lipids with a decrease in mRNA expression of CD36, ApoB48, FATP4, in the small intestine in mice. In the liver, activities of both fatty acid synthase and β-oxidation were downregulated by D-allulose to that of the normal group; however, in WAT, fatty acid synthase was decreased while β-oxidation activity was enhanced.
Conclusion: Taken together, our findings suggest that 5% dietary D-allulose led to the normalization of the metabolic status of diet-induced obesity by altering lipid-regulating enzyme activities and their gene-expression level along with fecal lipids.

Keywords:
D-allulose / Fecal lipid excretion / Lipogenesis / β-oxidation

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1 Introduction

Obesity is caused by an energy imbalance between caloric intake and consumption and is a major health problem worldwide. Over several decades, there has been a global shift in diet toward high energy intake due to energy-dense foods that are rich in fats and sugars, as well as a shift toward a more sedentary lifestyle [1]. These drastic changes in diet and lifestyle over several decades are considered the main promoters of obesity. The prevalence of obesity has now reached epidemic proportions and is frequently associated with multiple metabolic abnormalities including insulin resistance, glucose intolerance, and dyslipidemia [2–5]. These metabolic

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Abbreviations: **FAS**, fatty acid synthase; **ACC1**, acetyl-coA carboxylase1; **CPT 1α**, Carnitine palmitoyltransferase 1α; **CPT2**, Carnitine palmitoyltransferase 2; **CD36**, cluster of differentiation 36; **FATP4**, fatty acid transporter4; **ApoB48**, apolipoprotein B 48; **ABCG5**, ATP-binding cassette sub-family G member 5; **ABCG8**, ATP-binding cassette sub-family G member 8.

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abnormalities often occur concurrently and collectively underpin metabolic syndrome, a major contributor to morbidity and mortality [6].

D-allulose, a C-3 epimer of D-fructose, is a sugar substitute, which has 70% of the sweetness of sucrose but almost zero calories and is rarely found in nature [7, 8]. However, it is present in small quantities in commercial mixtures of D-glucose and D-fructose obtained from the hydrolysis of sucrose or the isomerization of D-glucose. It can now be manufactured in large quantities from the hydrolysis of sucrose or isomerization of D-glucose using enzyme methods of construction [9]. Regarding its safety matter, D-allulose is generally recognized as safe (GRAS) based on USDA regulations. Several studies have provided preliminary evidence on the impact of D-allulose on lipid metabolism. For example, D-allulose was suggested to act in a similar manner to D-fructose, however, it enhances glucose uptake from liver and suppresses hepatic lipogenic enzyme activity [10]. In addition, D-allulose appears to reduce intra-abdominal fat accumulation and suppresses the blood glucose rise in glucose loading in human subjects [11]. Interestingly, it lowered food intake while increasing energy expenditure in the light period and fat oxidation in the dark period in rats [12]. Most studies reported so far indicate that D-allulose induces a decrease in body weight and fat mass as well as food or energy intake in experimental animal models [10, 12].

This is the very first study investigating the anti-obesity effects of D-allulose in isocaloric pair-fed mice, thereby minimizing differences in food and energy intake between experimental groups. We hypothesized that dietary D-allulose at a 5% level is able to normalize the metabolic status of body weight and body fat mass by altering lipid metabolism in mice under isocaloric high fat-fed condition. Efficacy of D-allulose was compared with other sugar substitutes.

2 Material and methods

2.1 Animals and diets

A total of 60 male C57BL/6J mice (4 wk old) were purchased from the Jackson Laboratory (Bar Harbor, USA). The animals were maintained in a room with controlled temperature (20-23°C) and lighting (alternating 12-h periods of light and dark) and fed a pelletized commercial nonpurified diet for 1 week after arrival. The mice were then randomly divided into six groups ($n = 10$) and fed the respective experimental diets for 16 wk, as shown in Supplementary Table 1: normal diet control (ND, American Institute of Nutrition AIN-76 semisynthetic diet), high-fat diet control (HFD, 20% fat plus 1% cholesterol based on the AIN-76 diet), 5% D-allulose (ALL, 5% D-allulose substituted for sucrose in HFD, w/w), 5% erythritol (ERY, 5% erythritol substituted for sucrose in HFD, w/w), 5% D-glucose (GLU, 5% D-glucose substituted for sucrose in HFD, w/w,), and 5% D-fructose (FRU, 5% D-fructose substituted for sucrose in HFD, w/w). D-allulose was supplied by CJ CheilJedang Corp. (Seoul, Korea), and erythritol, D-glucose and D-fuctose were purchased from sigma Aldrich (Saint Louis, USA). The HFD was formulated to provide 39.5% of the total energy from fat, by replacing carbohydrate energy with lard and corn oil, and had the same amount of vitamins and minerals per kJ as the ND. The ALL group was given its D-allulose diet and other groups (HFD, ERY, GLU, and ERY) were given isocaloric diets based on the energy intake of the ALL group in a pair-fed manner. Mice had free access to distilled water during the experimental period. Their food intake was recorded daily, and body weights were monitored every 2 wk. All animal procedures were approved by the Ethics Committee for animal studies at Kyungpook National University, Republic of Korea (Approval No. KNU-2013-18).

2.2 Plasma, hepatic, and fecal lipid profile analysis

Plasma-free fatty acid, phospholipid, apolipoprotein AI (Apo A-1), and apolipoprotein B (Apo B) levels were measured using a Nittobo enzymatic kit (Nittobo Medical Co., Tokyo, Japan), and plasma HDL-cholesterol, triglyceride (TG) and total cholesterol (total-C) levels were determined using Asan enzymatic kits (Asan, Seoul, South Korea).

Hepatic and fecal lipids were extracted [13], and the dried lipid residues were dissolved in 1 mL of ethanol for TG, cholesterol, and fatty acid assays. Triton X-100 and a sodium cholate solution in distilled water were added to 200 μL of the dissolved lipid solution for emulsification. The TG, cholesterol, and fatty acid contents were analyzed with the same enzymatic kit used for the plasma analysis.

2.3 Lipid-regulating enzyme activity measurement

Samples were prepared and analyzed according to the method reported by Hulcher & Oleson [14]. Briefly, fatty acid synthase (FAS) activity was determined with a spectrophotometric assay according to the method by Nepokroeff et al. [15]. One unit of FAS activity represented the oxidation of 1 nmol of NADPH per minute at 30°C. The fatty acid β-oxidation was determined using the method by Lazarow [16] by monitoring the reduction of NAD to NADH at 340 nm, where the activity was implied by the reduced NAD nmol/min/mg protein. Carnitine palmitoytransferase (CPT) activity was determined according to the method of Markwell et al. [17].

2.4 Plasma leptin, resistin, and adiponectin measurement

Plasma leptin, resistin, and adiponectin were determined with a multiplex detection kit from Bio-Rad (Hercules, CA, USA). All samples were assayed in duplicate and analyzed with a Luminex 200 labmap system (Luminex, Austin, TX, USA). Data analyses were done with Bio-Plex Manager software version 4.1.1 (Bio-Rad, Hercules, CA, USA).

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2.5 Real-time qPCR analysis

Samples were prepared and analyzed as previously described [18]. The total RNA was converted to cDNA using the QuantiTect Reverse Transcription kit (QIAGEN Gmblh, Hilden, Germany). The RNA expression was quantified by real-time quantitative PCR using the QuantiTect SYBR Green PCR kit (QIAGEN Gmblh, Hilden, Germany) and the SDS7000 sequence-detection system (Applied Biosystems, CA, USA). Primers were designed to detect fatty acid synthase (FAS, 14101), acetyl-coA carboxylase 1 (ACC1, 107476), acetyl-coA carboxylas (ACC2, 700705), carnitine palmitoyltransferase 1α (CPT1α, 12894), carnitine palmitoyltransferase 2 (CPT2, 12896), peroxisome proliferator activated receptor alpha(Ppara, 19013), cluster of differentiation 36 (CD36, 12491), apolipoprotein B 48 (ApoB48, 238055), fatty acid transporter 4 (FATP4, 26569), ATP-binding cassette sub-family G member 5 (ABCG5, 27409), and ATP-binding cassette sub-family G member 8 (ABCG8, 67470). The amplification was performed as follows: 10 min at 90°C, 15 s at 95°C, and 60 s at 60°C, for a total of 40 cycles. The cycle threshold values were normalized using GAPDH. Relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method [19].

2.6 Histopathology analysis

Liver and epididymal white adipose tissue (WAT) were removed from mice and fixed in a buffer solution of 10% formalin. All fixed tissues were processed routinely for paraffin embedding and 4 mm sections were prepared and stained with hematoxylin and eosin. Stained areas were viewed using an optical microscope (Nikon, Tokyo, Japan) with a magnifying power of ×200.

2.7 Statistical analysis

All data were presented as the mean and standard error. Statistical analysis was performed using software SPSS (version 11.0, SPSS, Inc., Chicago, IL, USA). Statistical differences between ND and HFD results were determined using Student's *t*-test. One-way ANOVA was performed to compare HFD groups and a Turkey's multiple-range test was performed when significant differences were identified between the groups at $p < 0.05$.

3 Results

3.1 Effects of D-allulose on body weight and organ weight

To investigate the effects of long-term D-allulose supplementation in diet-induced obese mice, 5-week-old male C57BL/6J mice were provided with an HFD with or without sugar substitutes for 16 wk. The D-allulose group was compared with the ND control and other sugar substitute groups. Three sugar substitutes, ERY, GLU, and FRU, replaced the sugar in the HFD. As stated in the methods section, all groups, except the ND group were fed isocaloric diets. Body weight was markedly lower in mice fed an HFD supplemented with D-allulose compared to the HFD control group and HFD with other monosaccharides from week 8 (Fig. 1). Among all sugar-substituted groups, D-allulose supplementation only suppressed body weight gain and the weight gain pattern during the 16 wk was close to that of the ND group from week 4. As shown in Table 1, the final body weight, as well as weight gain of the ALL group, was significantly lower than that of ERY, GLU, and FRU groups. For these reasons, the food efficiency ratio of D-allulose group was significantly lower than HFD and other sugar-substituted groups.

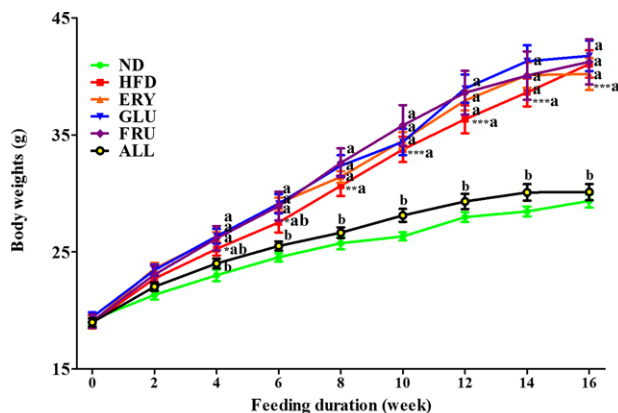

Figure 1. Changes of the body weight in C57BL/6J mice fed high-fat diet with supplementation of D-allulose, erythritol, d-glucose, and d-fructose for 16 wk. Data are mean ± SE; ND, normal diet (AIN-76); HFD, high fat diet (AIN-76, 20 % fat, 1% cholesterol); ALL, (HFD+5% D-allulose); ERY (HFD+5% Erythritol); GLU, (HFD+5% D-Glucose); FRU, (HFD+5% fructose); a,b,cMean values with different superscript letter are significantly different among HFD-fed groups ($p < 0.05$); Mean values are significantly different for ND from those of HFD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Organ weights were measured to determine whether this was the cause of body weight reduction. Weights of muscle and kidney were significantly lower, whereas liver weight was markedly higher in the HFD control group than in the ND group (Table 1). However, those of the ALL group were similar to values of the ND group. As indicated by Table 1, HFD feeding resulted in a significant increase in weight of visceral WATs including the perinephric, epididymal, retroperitoneum, and mesentery depots, interscapular brown adipose tissue (BAT), and total WAT compared to ND group, while all types of adipose deposits were significantly reduced in the ALL group.

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Table 1. Effects of D-allulose supplementation for 16 wk on body weight, food efficiency ratio, and organ and adipose tissue weights in C57BL/6J mice fed HFD

	ND	HFD	ERY	GLU	FRU	ALL
Initial bodyweight (g)	19.20 ± 0.37	18.97 ± 0.36	19.00 ± 0.54	19.45 ± 0.42	19.08 ± 0.59	18.99 ± 0.35
Final bodyweight (g)	29.37 ± 0.58	39.38 ± 1.40***a)	40.22 ± 1.36a)	41.77 ± 1.31a)	41.26 ± 1.93a)	30.13 ± 0.69b)
BWG (g/16wk)	0.64 ± 0.0.02	1.28 ± 0.08***a)	1.32 ± 0.10a)	1.39 ± 0.07a)	1.39 ± 0.09a)	0.70 ± 0.04b)
Food intake (g/day)	3.12 ± 0.10	3.03 ± 0.25	3.17 ± 0.26	3.03 ± 0.25	3.03 ± 0.25	3.17 ± 0.26
Energy intake (kcal/day)	12.16 ± 0.39	13.88 ± 1.15	13.88 ± 1.15	13.88 ± 1.15	13.88 ± 1.15	13.88 ± 1.15
FER	0.029 ± 0.001	0.060 ± 0.002***a)	0.059 ± 0.003a)	0.065 ± 0.003a)	0.065 ± 0.003a)	0.031 ± 0.002b)
Organ weight (g/100 g body weight)						
Muscle	1.08 ± 0.02	0.85 ± 0.03***b)	0.77 ± 0.03b)	0.82 ± 0.03b)	0.83 ± 0.04b)	1.01 ± 0.02a)
Liver	3.41 ± 0.05	5.49 ± 0.32***a)	5.37 ± 0.25a)	5.22 ± 0.15a)	5.68 ± 0.35a)	4.35 ± 0.08b)
Kidney	0.98 ± 0.02	0.81 ± 0.04*b)	0.77 ± 0.04b)	0.74 ± 0.02b)	0.84 ± 0.05b)	1.03 ± 0.02a)
Adipose tissue weight (g/100 g body weight)						
Perinephric fat	0.43 ± 0.04	1.25 ± 0.09***a)	0.92 ± 0.10a)	1.15 ± 0.19a)	1.17 ± 0.14a	0.38 ± 0.04b
Epididymal fat	3.04 ± 0.21	8.28 ± 0.61a)	7.92 ± 0.60a	8.42 ± 0.88a)	8.50 ± 0.71a)	3.67 ± 0.45b)
Retroperitoneum fat	0.88 ± 0.07	2.32 ± 0.08***a	2.21 ± 0.15a)	2.01 ± 0.22a,b)	2.19 ± 0.22a)	1.34 ± 0.32b)
Subcutaneous fat	1.61 ± 0.13	4.95 ± 0.41***a,b)	4.88 ± 0.15a,b)	5.47 ± 0.47a)	3.78 ± 0.84b)	1.99 ± 0.19c)
Mesentery fat	1.16 ± 0.06	2.95 ± 0.23***a)	2.43 ± 0.26a)	2.62 ± 0.44a)	2.93 ± 0.34a)	0.87 ± 0.09b)
Visceral fat	7.13 ± 0.48	19.74 ± 0.28***a)	18.36 ± 0.54a)	19.67 ± 0.41a)	18.22 ± 0.42a)	8.26 ± 0.17b)
Interscapular WAT	1.54 ± 0.10	4.42 ± 0.04***a)	4.56 ± 0.04a)	4.54 ± 0.05a)	4.43 ± 0.05a)	1.70 ± 0.02b)
Interscapular BAT	0.34 ± 0.02	0.75 ± 1.09***a)	0.57 ± 1.66a,b)	0.71 ± 1.52a)	0.73 ± 1.98a)	0.34 ± 0.98b)
Total WAT	9.01 ± 0.59	24.16 ± 1.34***a)	22.93 ± 2.20a)	24.21 ± 1.83a)	22.65 ± 2.37a)	9.95 ± 1.15b)

Data are mean ± SE; ND, normal diet (AIN-76); HFD, high fat diet (AIN-76, 20 % fat, 1% cholesterol); ALL, (HFD+5% D-allulose); ERY (HFD+5% Erythritol); GLU, (HFD+5% D-Glucose); FRU, (HFD+5% fructose).
a,b,c) Means values with different superscript letters are significantly different among HFD-fed groups ($p < 0.05$); Mean values are significantly different for ND from those of HFD, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.
BWG, body weight gain; FER, food efficiency ratio = body weight gain/food intake.

3.2 Effect of D-allulose on plasma lipid profiles

D-allulose supplementation altered the plasma lipid profiles in mice fed HFD. Initial plasma TG concentration was approximately the same in all six groups (Fig. 2). Although there were some fluctuations in plasma TG level during first 8 wk, its final concentration was lowest in the ALL and ND groups. Plasma total-C concentration was significantly higher in the HFD group compared to ND-fed mice from week 2. Among sugar-substituted groups, D-allulose suppressed the elevation of plasma total-C toward the ND group over the 16 wk (Fig. 2). At the end of the 16 wk, the concentration of FFA and

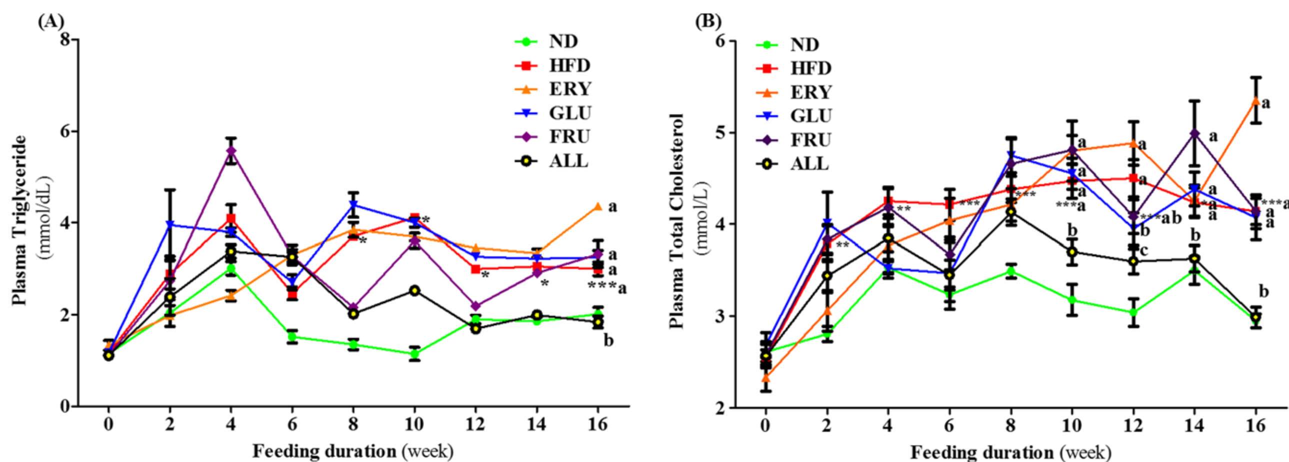

Figure 2. Changes of plasma (A)triglyceride and (B)total-cholesterol concentrations in C57BL/6J mice fed high-fat diet with supplementation of D-allulose, erythritol, d-glucose, and d-fructose for 16 wk. Data are mean ± SE; ND, normal diet (AIN-76); HFD, high fat diet (AIN-76, 20 % fat, 1% cholesterol); ALL, (HFD+5% D-allulose); ERY (HFD+5% Erythritol); GLU, (HFD+5% D-Glucose); FRU, (HFD+5% fructose); a,b,cMeans values with different superscript letter are significantly different among HFD-fed groups(p <0.05); Mean values are significantly different for ND from those of HFD, *p <0.05, **p <0.01, ***p <0.001.

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Table 2. Effects of D-allulose supplementation for 16 wk on plasma lipid profiles and glucose levels in C57BL/6J mice fed HFD

	ND	HFD	ERY	GLU	FRU	ALL
FFA (mmol/L)	0.38 ± 0.04	$0.52 \pm 0.05^{*a)}$	$0.53 \pm 0.02^{a)}$	$0.49 \pm 0.06^{a)}$	$0.48 \pm 0.04^{a)}$	$0.41 \pm 0.02^{b)}$
TG (mmol/L)	0.84 ± 0.06	$1.28 \pm 0.06^{***a)}$	$1.25 \pm 0.07^{a)}$	$1.28 \pm 0.08^{a)}$	$1.17 \pm 0.03^{a)}$	$0.99 \pm 0.04^{b)}$
PL (mg/dL)	88.64 ± 8.51	$115.58 \pm 5.28^{**b)}$	$135.46 \pm 6.28^{a)}$	$104.86 \pm 6.92^{b)}$	$115.27 \pm 5.85^{b)}$	$107.23 \pm 2.82^{b)}$
Total-C (mmol/L)	2.95 ± 0.17	$4.17 \pm 0.17^{***b)}$	$5.35 \pm 0.98^{a)}$	$4.07 \pm 0.17^{b)}$	$4.14 \pm 0.29^{b)}$	$2.99 \pm 0.09^{c)}$
HDL-C (mmol/L)	0.91 ± 0.04	$1.16 \pm 0.06^{*a)}$	$1.22 \pm 0.08^{a)}$	$0.82 \pm 0.03^{b)}$	$0.83 \pm 0.09^{b)}$	$1.28 \pm 0.07^{a)}$
non-HDL-C (mmol/L)	2.01 ± 0.15	$3.00 \pm 0.15^{***b)}$	$4.36 \pm 0.73^{a)}$	$3.25 \pm 0.15^{b)}$	$3.32 \pm 0.29^{b)}$	$1.84 \pm 0.13^{c)}$
LDL-C (mmol/L)	1.60 ± 0.08	$2.46 \pm 0.13^{***a)}$	$3.35 \pm 0.72^{a)}$	$2.61 \pm 0.12^{a)}$	$2.60 \pm 0.56^{a)}$	$1.45 \pm 0.56^{b)}$
ApoA-I (mg/dL)	26.41 ± 1.01	$28.62 \pm 0.85^{*b)}$	27.28 ± 0.58^{b}	$27.23 \pm 0.95^{b)}$	$27.69 \pm 0.48^{b)}$	$29.39 \pm 0.95^{a)}$
ApoB (mg/dL)	7.35 ± 0.89	$9.16 \pm 0.4^{*a,b)}$	$8.61 \pm 0.90^{b)}$	$9.32 \pm 1.04^{a,b)}$	$9.96 \pm 0.79^{a)}$	$7.63 \pm 0.52^{c)}$
AI	2.25 ± 0.15	$2.65 \pm 0.16^{a,b)}$	$3.55 \pm 0.13^{a,b)}$	$3.76 \pm 0.15^{a,b)}$	$4.55 \pm 0.66^{a)}$	$1.42 \pm 0.13^{b)}$
HTR	31.20 ± 1.37	$28.12 \pm 1.18^{b)}$	23.95 ± 3.53^{b}	$20.19 \pm 0.60^{b)}$	$20.62 \pm 2.56^{b)}$	$42.98 \pm 2.62^{a)}$
APOA-I/ APOB	3.59 ± 0.56	$3.12 \pm 0.17^{*b)}$	$3.16 \pm 0.34^{b)}$	$2.92 \pm 0.35^{b)}$	$2.77 \pm 0.21^{b)}$	$3.80 \pm 0.23^{a)}$
Glucose (mmol/L)	9.70 ± 0.62	$15.81 \pm 0.78^{***a)}$	$14.74 \pm 0.42^{a)}$	$15.16 \pm 0.66^{a)}$	$16.12 \pm 0.57^{a)}$	$10.08 \pm 0.36^{b)}$

Data are mean $\pm$ SE; ND, normal diet (AIN-76); HFD, high fat diet (AIN-76, 20 % fat, 1% cholesterol); ALL, (HFD+5% D-allulose); ERY (HFD+5% Erythritol); GLU, (HFD+5% D-Glucose); FRU, (HFD+5% fructose).
a,b,c) Mean values with different superscript letters are significantly different among HFD-fed groups ($p < 0.05$); Mean values are significantly different for ND from those of HFD, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.
TG, triglyceride; **C**, cholesterol; **PL**, phospholipid; **HDL-C**, high-density lipoprotein; **LDL-C**, low-density lipoprotein; **APOA-I**, apolipoprotein A-I; **Apo B**, apolipoprotein B; **HTR**, (HDL-C/Total-C)$\times$100; **AI**, Atherogenic index, [(Total-C)-(HDL-C)]/HDL-C

non-HDL-C were also significantly lowered by D-allulose supplementation compared to the HFD group (Table 2). However, the levels of phospholipid (PL) and HDL-cholesterol (HDL-C) of the ALL group were not different from the HFD group. In the ALL group, HTR was concomitantly elevated due to decreased plasma total-C concentration. In contrast to D-allulose, ERY supplementation even elevated the plasma total-C and non-HDL-C compared to the HFD group, and the HDL-C concentration was lower in GLU and FRU groups than in the HFD group. Apo A-1 concentration was significantly higher in only the ALL group, whereas the Apo-B concentration was only decreased by ALL toward the ND group value.

3.3 Effects of D-allulose on plasma leptin, resistin, and adiponectin levels and the leptin:adiponectin ratio

As shown in Fig. 3, levels of plasma leptin, resistin, and the leptin:adiponectin ratio were drastically higher in the HFD control group compared to the ND group. However, supplementation of D-allulose led to a reduction in these values.

3.4 Effects of D-allulose on hepatic lipid profiles, tissue morphology, and lipid-regulating enzyme activities

As shown in Fig. 4A, hepatic triglyceride, cholesterol, and fatty acid content was significantly higher in the HFD group than in the ND group. However, when D-allulose was supplemented with HFD, the hepatic lipid content was significantly lowered toward the ND group, unlike other sugar-substituted groups. ERY supplementation even induced the elevation of hepatic TG compared to the HFD group. Hematoxylin and eosin staining of liver sections indicated that hepatic lipid accumulation was more pronounced in the HFD group compared to ND-fed mice (Fig. 4C). Interestingly, supplementation of D-allulose resulted in a reduction of lipid droplet numbers and cell size compared to the HFD control and other sugar substitute-supplemented groups.

Among lipid-regulating hepatic enzymes, as indicated by (Fig. 4B), activities of FAS and PAP, which are rate-limiting enzymes for FA and TG synthesis, respectively, were significantly elevated in the HFD group compared to the ND group. However, ALL resulted in significant decreases in these enzyme activities toward to ND group level. The activity of hepatic β-oxidation was significantly lowered toward that of the ND group in the ERY, GLU, and ALL groups.

3.5 Effects of D-allulose on adipose tissue morphology and lipid-regulating enzyme activities

Hematoxylin and eosin staining of epididymal WAT sections indicated that expansion of fat cell sizes were pronounced in the HFD, ERY, FRY, and GLU groups compared to the ND group (Fig. 5C). However, the epididymal adipocyte size was reduced relatively in the D-allulose-supplemented mice.

The activity of FAS in epididymal WAT was significantly elevated while β-oxidation activity was diminished in HFD-fed mice compared to the ND group (Fig. 5A). However, D-allulose led to a significant decrease in FAS activity with a concomitant increase in β-oxidation activity and CPT, whereas FAS activity was increased in the other sugar substitute groups. Also, D-allulose significantly increased the mRNA expression of CPT1a and CPT2 and significantly decreased the mRNA expression of ACC2 (Fig. 5B).

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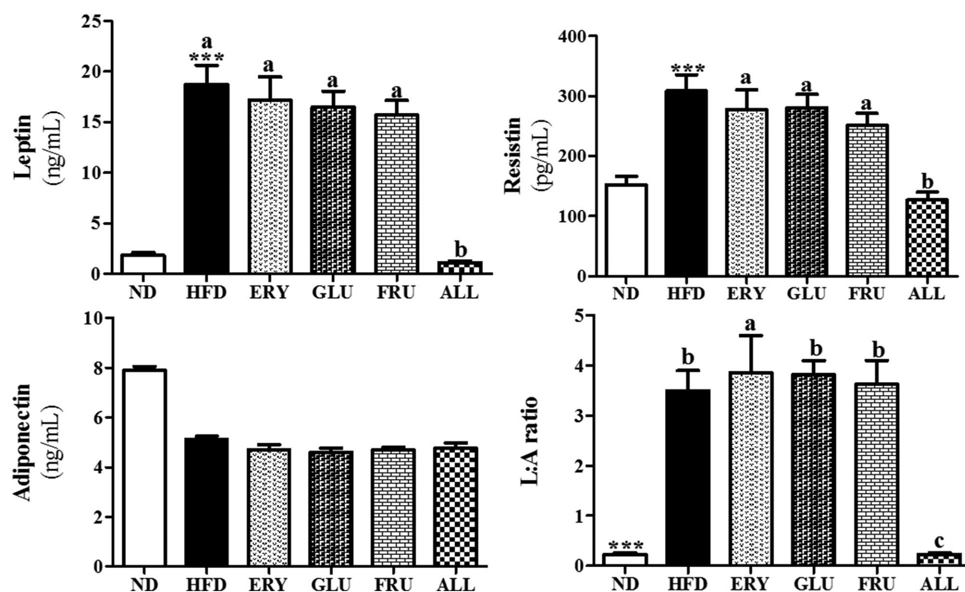

Figure 3. Effect of D-allulose, erythritol, D-glucose, and D-fructose supplement on leptin, resistin, and adiponectin levels and L:A ratio in C57BL/6J mice fed high-fat diet for 16 wk. Data are mean ± SE; ND, normal diet (AIN-76); HFD, high fat diet (AIN-76, 20 % fat, 1% cholesterol); ALL, (HFD+5 % D-allulose); ERY (HFD+5 % Erythritol); GLU, (HFD+5 % D-Glucose); FRU, (HFD+5 % fructose); [a,b,c]Mean values with unlike superscript letter are significantly different (p <0.05); Mean values were significantly different for ND from those of HFD, $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001. L:A ratio, leptin:adiponectin ratio.

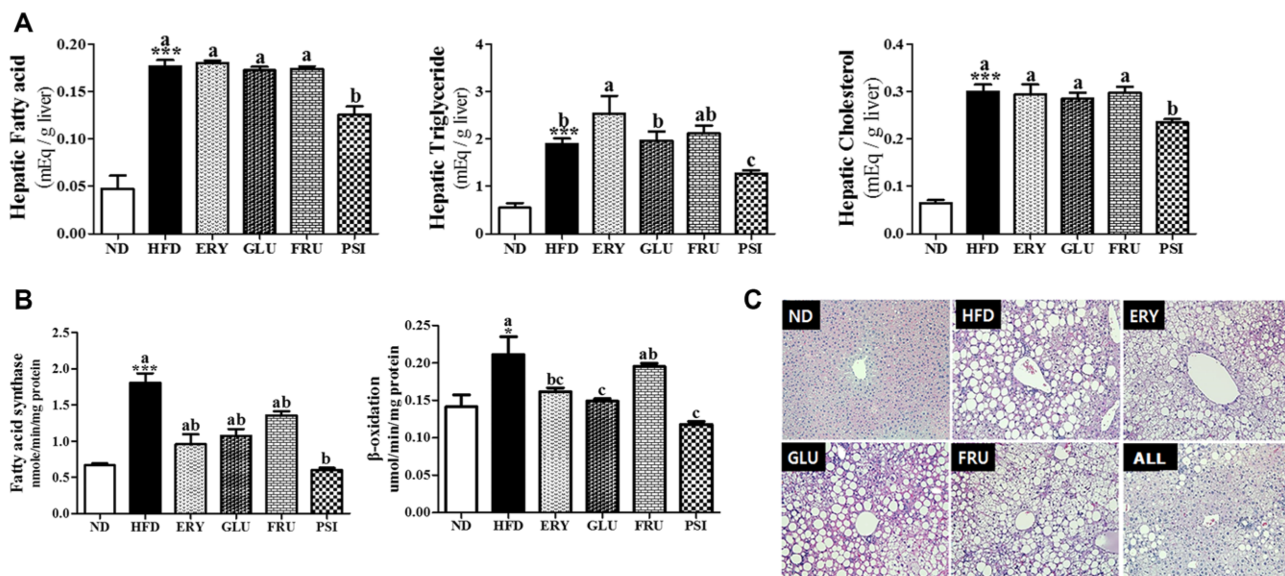

Figure 4. Effects of D-allulose, erythritol, d-glucose, and d-fructose supplement on hepatic (A) lipids levels, (B) activities of FAS, and β-oxidation and (C) morphology in C57BL/6J mice for 16 wk. Data are mean ± SE; ND, normal diet (AIN-76); HFD, high fat diet (AIN-76, 20 % fat, 1% cholesterol); ALL, (HFD+5% D-allulose); ERY (HFD+5% Erythritol); GLU, (HFD+5% D-Glucose); FRU, (HFD+5% fructose); [a,b,c]Mean values with different superscript letter are significantly different among HFD-fed groups (p <0.05); Mean values are significantly different for ND from those of HFD, *p <0.05, **p <0.01, ***p <0.001. Hematoxylin and eosin (H&E) stained transverse section of liver. Representative photomicrographs of liver are shown at ×200 magnification.

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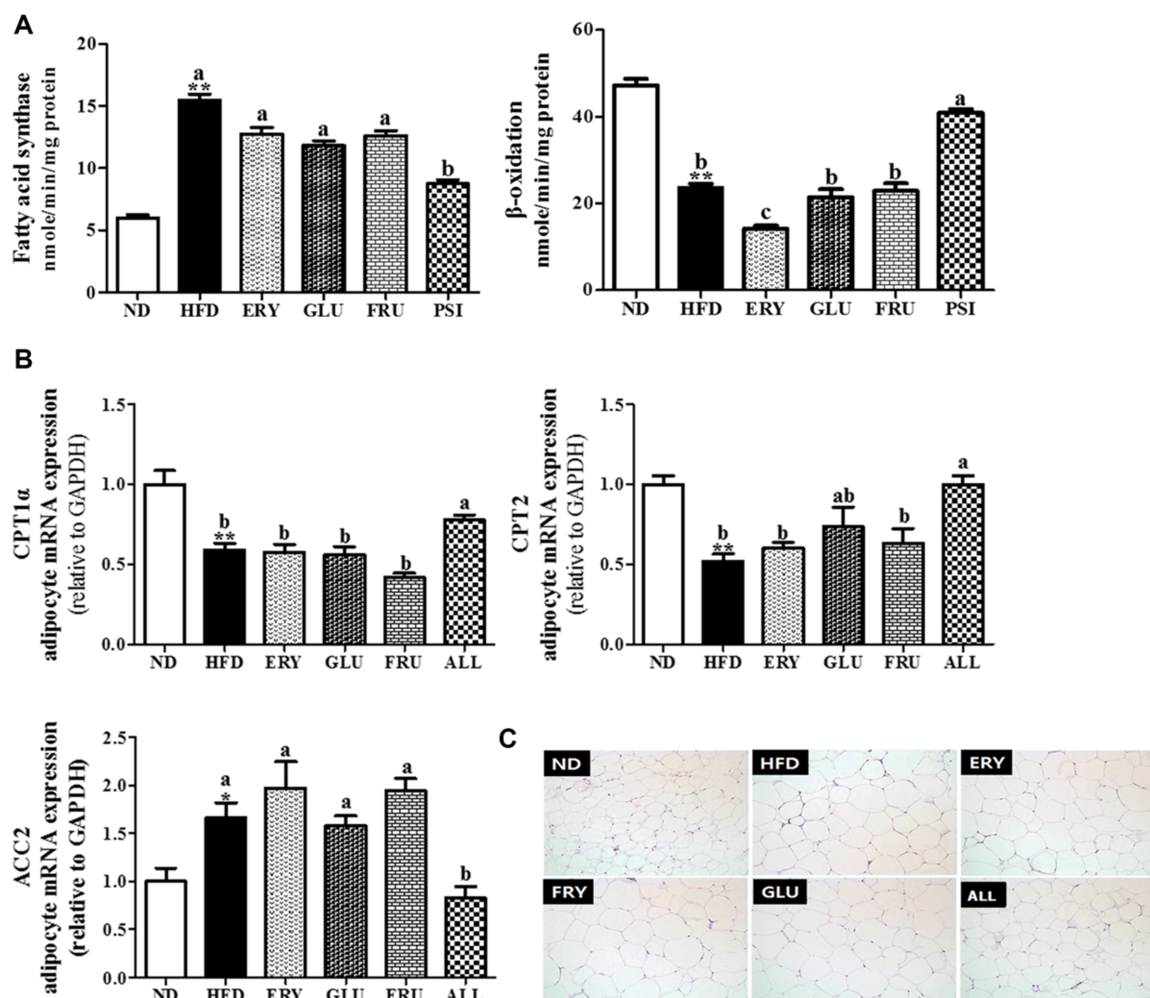

Figure 5. Effect of D-allulose, erythritol, d-glcucose, and d-fructose supplementation on the adipose tissue (A)activities of FAS β-oxidation and CPT (B) mRNA expression of CPT1α, CPT2, and ACC2 and (C) morphology in C57BL/6J mice for 16 wk. Data are mean ± SE; ND, normal diet (AIN-76); HFD, high fat diet (AIN-76, 20% fat, 1% cholesterol); ALL, (HFD+5% D-allulose); ERY(HFD+5% Erythritol); GLU, (HFD+5% d-Glucose); FRU, (HFD+5% fructose); ^{a,b,c}Mean values with different superscript letters are significantly different among HFD-fed groups (p <0.05); Mean values are significantly different for ND from those of HFD, *p <0.05, **p <0.01, ***p <0.001. CPT 1α, carnitine palmitoyltransferase 1α; CPT2, carnitine palmitoyltransferase 2; ACC2, acetyl-coA carboxylase 2. Hematoxylin and eosin (H&E) stained transverse-section of epididymal fat. Representative photomicrographs of epididymal WAT are shown at ×200 magnification.

3.6 Effect of D-allulose on expression of hepatic genes related to fatty acid synthesis and oxidation

Changes in the expression of genes controlling lipogenesis and fatty acid oxidation in hepatic tissue were assessed in order to understand the molecular mechanisms underlying the inhibitory effect of D-allulose on hepatic fat accumulation (Fig. 6). The mRNA expression of hepatic lipogenic genes including FAS and ACC1 was significantly decreased in the HFD group based on the ND group. However, mRNA expression of these genes was further lowered by ALL supplementation compared to HFD or other groups. The mRNA expression of CPT1α, CPT2, and ACC2 was significantly lower in the HFD than in the ND group. Again, the mRNA expression of CPT1 α, CPT2 was significantly lowered and mRNA expression of ACC2 was significantly increased by D-allulose supplementation.

3.7 Effects of D-allulose on fecal lipid excretion and expression of genes involved in lipid metabolism of small intestine

Fecal TG, cholesterol, and FFA contents were significantly higher in all HFD-fed groups including sugar-substituted groups when compared to those of ND-fed groups ($p < 0.001$) (Fig. 7). However, D-allulose supplementation led to a further increase in fecal lipid excretion ($p < 0.001$).

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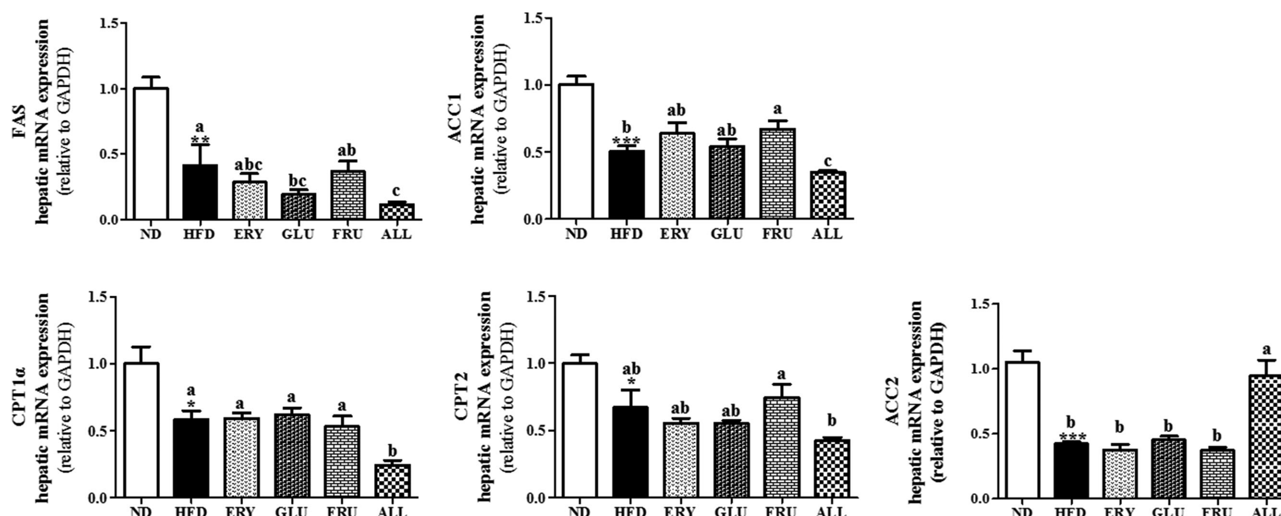

Figure 6. Effect of D-allulose, erythritol, D-glcucose, and D-fructose supplementation on mRNA expression of genes related to fatty acid synthesis and oxidation in liver of C57BL/6J mice fed high-fat diet for 16 wk. The mRNA levels were normalized by the GAPDH mRNA expression. Data are mean ± SE; ND, normal diet (AIN-76); HFD, high fat diet (AIN-76, 20 % fat, 1% cholesterol); ALL, (HFD+5% D-allulose); ERY (HFD+5% Erythritol); GLU, (HFD+5% D-Glucose); FRU, (HFD+5% fructose); a,b,cMean values with different superscript letters are significantly different among HFD-fed groups (p <0.05); Mean values are significantly different for ND from those of HFD, *p <0.05, **p <0.01, ***p <0.001. FAS, fatty acid synthase; ACC1, acetyl-coA carboxylase 1; CPT 1α, carnitine palmitoyltransferase 1α; CPT2, carnitine palmitoyltransferase 2; ACC2, acetyl-coA carboxylase 2.

Changes in mRNA expression of genes related to cholesterol transport and lipid excretion in the small intestine were assessed (Fig. 8). The mRNA expression of Ppara, CD36, ApoB48, and FATP4, intracellular lipid transporter, and distributor were significantly increased in the HFD group compared to the ND group, however, this gene expression was markedly downregulated toward the ND group value by D-allulose supplementation. The mRNA expression for abcg5 and abcg8 was significantly elevated in the HFD as well as in all sugar substitute groups compared to the ND group.

4 Discussion

There is a growing interest in the development of sugar substitutes because of their reduced- or zero-calorie nature. D-allulose has attracted much attention recently as a no-energy sweetener. However, regardless of energy, its physiological effects, and underlying mechanisms are not completely understood. In the present study, using C57BL/6J mice that are susceptible to diet-induced obesity and share characteristics of human obesity compared to genetically obese animals [20], we determined whether D-allulose can display normalization of lipid metabolism in diet-induced obese mice when supplemented at a 5% dietary level.

4.1 D-Allulose results in an increase in fecal lipid excretion and improves plasma lipid profile

Although the digestion of dietary fats begin in the mouth, where lingual lipase breaks down short chain lipids into

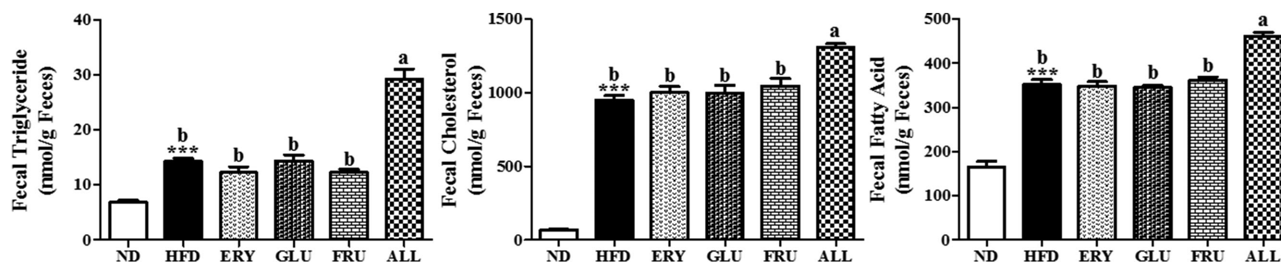

Figure 7. Effect of D-allulose, erythritol, d-glucose, and d-fructose supplement on fecal lipid excretion in C57BL/6J mice fed high fat diet for 16 wk. Data are mean ± SE; ND, normal diet (AIN-76); HFD, high fat diet (AIN-76, 20 % fat, 1% cholesterol); ALL, (HFD+5% D-allulose); ERY (HFD+5% Erythritol); GLU, (HFD+5% D-Glucose); FRU, (HFD+5% fructose); a,b,cMean values with different superscript letter are significantly different among HFD-fed groups (p <0.05); Mean values are significantly different for ND from those of HFD, *p <0.05, **p <0.01, ***p <0.001.

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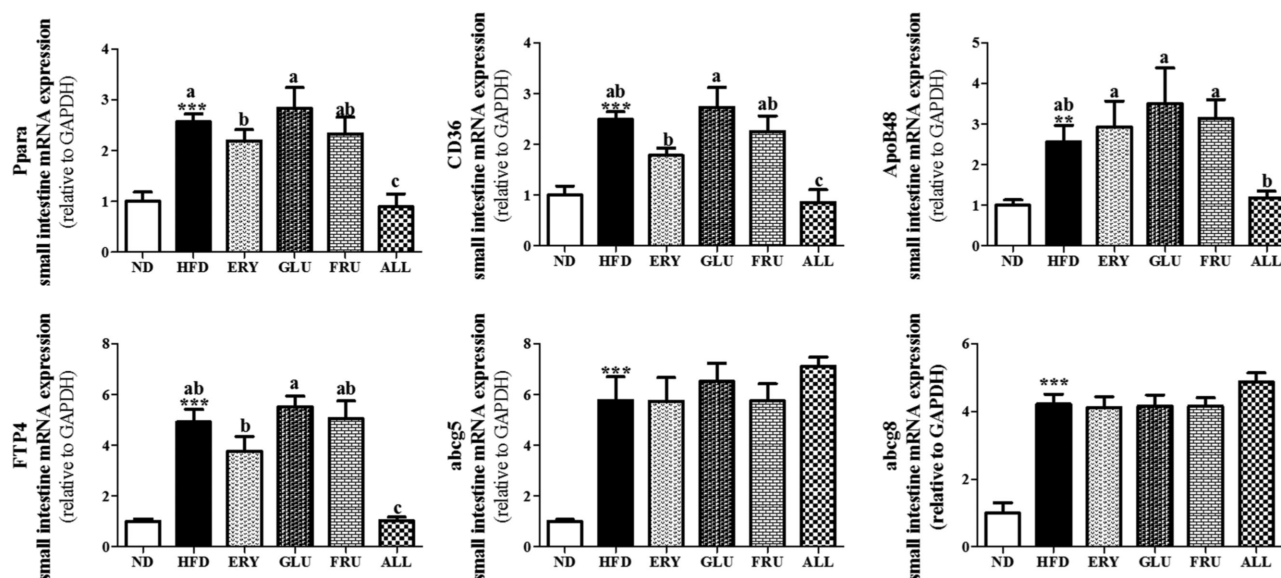

Figure 8. Effect of D-allulose, erythritol, d-glucose, and d-fructose supplementation on mRNA expressions of genes related to lipid excretion in small intestine of C57BL/6J mice fed high-fat diet for 16 wk. The mRNA levels were normalized by the GAPDH mRNA expression. Data are mean ± SE; ND, normal diet (AIN-76); HFD, high fat diet (AIN-76, 20 % fat, 1% cholesterol); ALL, (HFD+5% D-allulose); ERY (HFD+5% Erythritol); GLU, (HFD+5% D-Glucose); FRU, (HFD+5% fructose); [a,b,c]Mean values with different superscript letter are significantly different among HFD-fed groups ($p < 0.05$); Mean values are significantly different for ND from those of HFD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Ppara, peroxisome proliferator activated receptor alpha; CD36, CD36 antigen; FATP4, fatty acid transporter 4; ApoB48, apolipoprotein B48; ABCG5, ATP-binding cassette sub-family G member 5; ABCG8, ATP-binding cassette sub-family G member 8.

diglycerides, dietary fats are mostly digested in the small intestine [21]. Digestion of dietary fats results in glycerol, fatty acids, monoglyceride, free cholesterol, and other molecules. These are absorbed into the small intestinal epithelial cell lining in their own way.

Cluster of differentiation 36 (CD36), is known as a fatty acid translocase that can be expressed in the brush border membranes of the small intestine of rodent and be regulated by PPAR-α in small intestine. [22, 23]. It binds to a wide variety of lipid ligands, including lipoprotein, anionic PL, long chain FA, and cholesterol [22, 24, 25]. Its high expression and defined localization in the small intestine, together with its high affinity for lipids, suggest an important role of CD36 in lipid absorption [26]. When fatty acids are transported in small intestinal epithelial cell by FATP4, they are re-esterified with diglyceride to form triglyceride. The triglyceride is packed with phospholipids, cholesterol esters, and ApoB48 to form chylomicrons. In the small intestine, CD36 and FATP4 are responsible for fatty acid uptake and transport, respectively, to form chylomicrons, with ApoB48 being a major apoprotein component [27–29]. In the present study, mRNA expression of Ppara, CD36, FATP4, and ApoB48 in the small intestine was significantly decreased to that of the normal group levels as a result of D-allulose supplementation. Consistent with these results, supplementation of D-allulose led to marked increases in fecal triglyceride, FFA, and cholesterol levels, although intestinal SR-B1 was not measured, which is responsible for transport of free cholesterol into enterocytes. Accordingly, D-allulose significantly inhibited lipid absorption in the small intestine, thereby increasing fecal lipid excretion in HFD-fed mice. In addition, plasma lipid profiles were normalized by D-allulose supplementation. HFD feeding led to an increase in total-cholesterol concentration and a decrease in Apo A-1:Apo B ratio in plasma. ApoA-1 and Apo-B concentrations independently or together with their ratio of Apo A-1:Apo B predict cardiovascular risk and may be more accurate predictors than plasma lipids levels [30]. The D-allulose induced increase in the Apo A-1:Apo B ratio may have been due to the reduction of plasma total cholesterol and non-HDL cholesterol [30]. Although some limitations are present regarding no measurements in pancreatic lipase and acyl-CoA:cholesterol acyltransferase (ACAT) in small intestine, the alteration of these enzyme activities could affect digestion and absorption of dietary lipids and cholesterol. Taken together, the plasma lipid-lowering action of D-allulose is partly mediated by a decrease in intestinal lipid absorption.

4.2 D-allulose lowers fad-pad mass and inflammatory adipokines, and alters lipid-regulating enzymes activity in fat tissue

A combination of excessive caloric intake and the availability of an energy-dense diet are regarded as the main contributors to obesity [19]. HFD is high in energy density, thus elevates body weight due to increased adiposity in various rodent models [31]. In the present study, body weight, fat-pad mass, and adipocyte size were significantly increased in the

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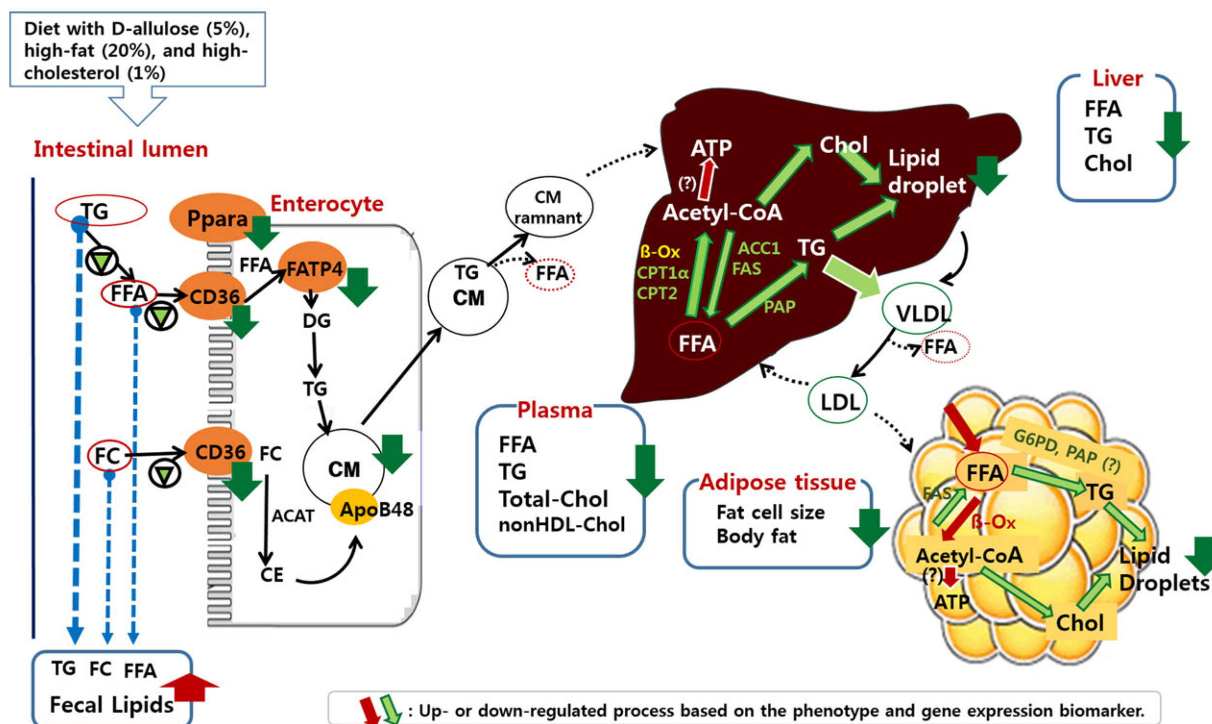

Figure 9. Proposed role of D-allulose supplementation on lipid metabolism in small intestine liver and adipose tissue based on present findings. FFA, free fatty acid; Chol, cholesterol; CE cholesteryl ester; FC, free cholesterol; TG, triglyceride; CM, chylomicron; ACAT, acyl-CoA: cholesterol acyltransferase; Ppara, peroxisome proliferator activated receptor alpha; CD36, cluster of differentiation 36; FATP4, fatty acid transporter 4; ApoB48, apolipoprotein B 48; ß-Ox, ß-oxidation.

HFD-fed mice, but were significantly diminished by D-allulose. In particular, D-allulose lowered visceral fats, which secrete inflammatory adipokines such as leptin and resistin. Additionally, morphological observations indicated that the epididymal adipocyte size was smaller in the D-allulose-supplemented mice compared to the HFD group. Similar to our results, D-allulose reduced body fat accumulation in rats fed an isocaloric high-sucrose diet [32]. In general, lipogenic enzyme activity is closely associated with diet-induced obesity. HFD led to increases in lipogenic enzyme activities in WAT, while D-allulose decreased the FAS activity. Our results are in accordance with part of another study in which 5% levels of dietary D-allulose reduced body fat accumulation, in rats, due to inhibition of lipogenic enzyme activity in adipose tissue compared to 5% fructose or 5% D-glucose diets; however, their food conversion efficiency was not different (glucose and fructose groups: 0.3, D-allulose group: 0.28) [10] while food efficiency ratio of D-allulose group in our study was lowest among groups (HFD group: 0.060, ERY group: 0.059, GLU group: 0.065, FRU group: 0.065, ALL group: 0.031). Activities of lipogenic enzymes were also significantly decreased in HFD-fed Sprague–Dawley rats with 3% D-allulose without pair-feeding compared to HFD-fed control groups [33]. In our study, D-allulose significantly increased β-oxidation and CPT activity in epididymal WAT that could inhibit lipid accumulation by inhibiting the re-esterification of FA to triglyceride and using acetyl-CoA from β-oxidation of FA as respiratory fuel [34]. Also, mRNA expressions of CPT1-α and CPT2 were significantly decreased and mRNA expression of ACC2 was significantly increased by D-allulose supplementation in present study. Therefore, D-allulose can inhibit lipid accumulation via inhibition of lipogenesis and activation of FA oxidation in epididymal WAT. The anti-adiposity effect of D-allulose was supported by the reduced levels of plasma leptin and resistin in our study, as these adipokine levels are positively correlated with body fat mas [35].

4.3 Proposed mechanism of D-allulose on normalizing lipid metabolic status in diet-induced obesity

ERY is a zero energy sweetener, as is D-allulose. Interestingly, the biological activity of D-allulose was entirely different from ERY or other monosaccharides in diet-induced obese mice. Based on our observations, the action of D-allulose could be proposed, shown in Fig. 9. D-allulose can inhibit intestinal lipid absorption that may decrease the TG availability for chylomicron formation. D-allulose led to increased utilization of FFA in adipose tissue toward β-oxidation by limiting its use for TG synthesis and lipid droplet formation that eventually led to the reduction of body fat and adipocyte size. However,

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liver synthesis of FFA and TG is low in the D-allulose group, based on its diminished activity of FAS and PAP, which eventually leads to a decrease in hepatic lipid accumulation and VLDL secretion.

Long-term consumption of an HFD is often associated with hepatic steatosis because excessive dietary fat leads to an accumulation of excess triglycerides in the liver [36]. Hepatomegaly, which is the condition of having an enlarged liver is one of the symptoms of hepatic steatosis. In the present study, ALL reduced liver weight and hepatic lipids compared to all HFD-fed mice. Furthermore, hepatic lipid droplet accumulation was less pronounced in the D-allulose supplemented group (Fig. 4). Also, D-allulose supplementation downregulated expression of fatty acid synthesis related genes in liver, such as FAS and ACC1, as well as genes involved in fatty acid oxidation, such as CPT1α and CPT2, compared to other HFD groups (Fig. 9). Interestingly, decreased hepatic CPT1α and CPT2 mRNA expression and increased hepatic ACC2 mRNA expression by D-allulose was consistant with decreased hepatic β-oxidation activity. Although further studies are needed to elucidate exact mode of action of D-allulose in detail, including hepatic cholesterol regulation and intestinal lipid absorption, it is clear that D-allulose can exert an inhibitory action on body fat acumulation and the initiation of hepatic steatosis or its progression. The suppression of hepatic lipogenesis by D-allulose was linked to decreased activities of hepatic FAS and PAP.

5 Conclusion

In our study, long-term supplementation of D-allulose appeared to reduce the body weight gain and reduce fat-pad mass. This is the very first study for a 5% D-allulose supplemented-HFD feeding compared with other monosaccharides supplemented-isocaloric HFD for 16 wk in C57BL/6J mice. Daily consumption of D-allulose, such as 5% (w/w) in the diet, was able to normalize the metabolic status of diet-induced obesity in these mice, which should be one of challenged scientific findings with a sugar substitute and functional food ingredient. It was markedly effective for protecting against HFD-induced obesity, hepatic steatosis, and dyslipidemia. It is plausible that these pathologies are mediated by alteration of lipid metabolic enzyme activities and/or gene expression in liver, adipose tissue and small intestine and by enhanced fecal lipid excretion. The present findings indicate that D-allulose can exert its biological effects on lipid metabolism regardless of its zero-energy density. Accordingly, D-allulose can be used as a functional monosaccharide or even as a medicinal food source for the prevention or treatment of obesity and related lipid disorders. As this compound has no toxic effect in vivo, human trials are expected with the appropriate dosage in the near future.

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The authors have declared no conflict of interest.

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