Beneficial Effects of Supplementation of the Rare Sugar "D-allulose" Against Hepatic Steatosis and Severe Obesity in *Lep^{ob}/Lep^{ob}* Mice

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Abstract: A rare sugar, D-allulose (also called D-psicose), has recently been applied as a food supplement in view of controlling diabetes and obesity in Japan. D-allulose has been proven to have unique effects against hyperglycemia and hyperlipidemia in a number of studies using several species of rats and mice. However, the antiobesity effects of D-allulose have not yet been assessed in *Lep^{ob}/Lep^{ob}* (*ob/ob*) mice. Therefore, this study explored the dietary supplemental effects of this sugar in leptin-deficient *ob/ob* mice. Consequently, the subchronic ingestion of D-allulose in *ob/ob* mice for 15 wk significantly decreased the body and liver weights, and the loss of body weight was involved in the reduction of the total fat mass, including abdominal visceral fat, and not fat-free body mass, including muscle. Furthermore, D-allulose improved hepatic steatosis, as evaluated using hepatic histological studies and MRI. In the normal mice, none of these parameters were influenced by the single or long-term ingestion of D-allulose. These results indicate that dietary supplementation of D-allulose especially influences postprandial hyperglycemia and obesity-related hepatic steatosis, without exercise therapy or dietary restriction. Therefore, D-allulose may be useful as a supplement for preventing and improving obesity and obesity-related disorders.

Keywords: D-allulose, dietary supplements, hepatic steatosis, obesity, sugar

Introduction

The rise in obesity is a major public health concern worldwide. Obesity is a common nutritional disorder, defined as an excessive overweight status presenting with a high body fat, often associated with numerous health problems. The prevalence of obesity in the Organization for Economic Cooperation and Development (OECD) countries is more than half of the adult population (53%) based on latest surveys (OECD Health Statistics 2012), and being overweight is often associated with type 2 diabetes as a result of insulin resistance (Saltiel 2001; Wang and others 2005). The rate of obesity is lowest in Japan (4.1% in 2011) and highest in the U.S.A. (36.5% in 2010), based on the WHO criteria among OECD member countries (Factbook Country Statistical Profiles in OECD 2014). However, the prevalence of obesity has also been rising in Japan due to the increased adoption of a westernized meal style and decreased physical activity. Although sugar has been a major component of the human diet since ancient times, a high intake of sugar may be associated with an increased risk of health conditions, such as obesity, cardiovascular disease, diabetes, gout, fatty liver, and dental caries (Bristol and others 1985; Milich and other 1986; Burt and Pai 2001; Johnson and others 2007; van Baak and Astrup 2009). In particular, the increasing intake of sugar-sweetened beverages, sweets, and desserts high in glucose and fructose has recently been identified to be a major contributor to the obesity epidemic (Ludwig and others 2001; Mozaffarian and others 2011; Te Morenga and others 2012). Therefore, decreasing the intake of sugar is necessary to achieve weight maintenance. However, it is difficult to strictly control the intake of sugar and/or sugar-containing foods and beverages. One approach that may be helpful is replacing sugar-sweetened items with products manufactured with artificial sweeteners that provide a sweet taste but with fewer calories.

"Rare sugars," monosaccharides, exist very rarely in nature, but small quantities are present in commercial mixtures of D-glucose and D-fructose obtained from the hydrolysis of sucrose or the isomerization of D-glucose (Cree and Perlin 1968). One such rare sugar is D-allulose (previously referred to as D-psicose), an epimer of D-fructose isomerized at C-3 position that is found in wheat, *Itea* plants, processed cane, and beet molasses (Matsuo and others 2001; Oshima and others 2006; Baek and others 2010). D-allulose has been proven to have antiobesity, antihyperlipidemic, and antihyperglycemic effects (Nagata and others 2015). Due to its rarity, there is limited knowledge regarding the biological functions of D-allulose. However, Izumori's group has recently established a new method for the large-scale production of rare sugars, including D-allulose (Takeshita and others 2000; Granstrom and others 2004), using the enzyme D-tagatose 3-epimerase (Itoh and others 1995). Following the mass production of D-allulose, several investigations have determined dramatic effects of D-allulose, both experimentally (Matsuo and Izumori 2006; Matsuo and Izumori 2009; Baek and oyhers, 2010; Hossain and others, 2012, Hossain

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and others 2015) and clinically (Iida and others 2008; Hayashi and others 2010), against obesity and type 2 diabetes mellitus (T2DM). These studies reflect the potential use of D-allulose as a substitute for sugar in foodstuffs in order to maintain the physiological levels of blood sugar and prevent excess fat deposition. Subsequently, D-allulose was approved as "generally recognized as safe" by the U.S. Food and Drug Administration in Aug, 2011 (GRN No. 400) and is allowed to be used as an ingredient in a wide range of foods and dietary supplements (Mu and others 2012).As potential mechanisms of controlling high glucose levels, the potency of D-allulose absorption over D-glucose in the intestine (Hishiike and others 2013) and the inhibition of enzymatic activities for the digestion of polysaccharides, such as glucoamylase and maltase, have been mentioned (Matsuo and Izumori 2006). D-allulose has also been shown to inhibit hepatic fatty acid synthatase (Matsuo and others 2001) as the mechanism of controlling adipose tissue deposition followed by decreased body weight gain. In addition to antiobese and antihyperglycemic effects of D-allulose its zero-calorie credit and 70% relative sweetness (Matsuo and others 2002) attracted food companies to prepare D-allulose-added various foodstuffs as a substitute of sugar. Based largely on these assn., many researchers and healthcare practitioners have proposed that noncaloric, high-intensity sweeteners provide a beneficial alternative in foods and beverages (Matsuo and Izumori 2006; Grandner and others, 2012) reported that supplemental D-allulose for 8 wk reduced body weight gain and abdominal fat mass in normal rats. Previously we reported that short-term administration of 5% D-allulose in the drinking water served as a unique metabolic regulator in growing Otsuka Long-Evans Tokushima Fatty (OLETF) rats with T2DM via the maintenance of blood glucose and prevention of abdominal fat deposition (Hossain and others 2011; Hossain and others 2012). More recently, we demonstrated that long-term administration of D-allulose also significantly maintained the body weight and blood glucose levels compared with diabetic controls (Hossain and others 2015). In both short- and long-term studies, other mechanisms included the preservation of pancreas β-cells through the suppression of proinflammatory cytokines and reactive oxygen species production. These results suggest that D-allulose may be a potential antidiabetic agent, even as an ingredient in food.

The inherited deficiency of leptin, an appetite-suppressing hormone, causes obesity, and obesity-related syndromes (Ingalls and others 1950; Mayer and others 1951; Herberg and Coleman 1977; Bray and York 1979; Zhang and others 1994). An inherited leptin-deficient *Lep^{ob}/Lep^{ob}* (*ob/ob*) mouse develops obesity-related hyperglycemia and hepatic steatosis with increased lipogenesis, has been reported in both the liver and adipose tissue (Herberg and Coleman 1977; Bray and York 1979). Montague and others (1997) also showed that *ob/ob* mice presented the most severe obesity ever shown in both rodents and humans. Therefore, these animals provide a good model of obesity and related syndromes, including glucose intolerance, insulin tolerance, and fatty liver disease.

In the present study, we examined the effects of the subchronic ingestion of D-allulose on obesity and hepatic steatosis in *ob/ob* mice. In addition, we performed *in vivo* evaluations with the goal of characterizing the morphological aspects of adipose tissues and other visceral organs using magnetic resonance imaging (MRI). This study is the first to examine the dietary supplemental benefits of D-allulose in inherited leptin-deficiency mice with severe obesity, which particularly influences the rate of obesity and development of leptin-deficient-dependent hepatic steatosis.

Materials and Methods

Animals

Two groups of *Lep^{ob}/Lep^{ob}* (*ob/ob*) and wild-type (WT) C57BL/6J mice, obtained from Charles River Lab. Intl, Inc. (Osaka, Japan) were used. All mice were housed at a constant temperature (24 °C) on a 12-h light/dark cycle and fed standard mouse chow ad libitum. The protocols for all animal experiments were approved by the Tokushima Bunri Univ. Animal Care Committee according to the National Institutes of Health (U.S.A.) Animal Care and Use Protocol. All efforts were made to minimize the number of animals used and avoid their suffering.

Food and water intake

The animals were allowed free access to both water and food (pellets). In the subchronic studies, the quantity of food and drink intake was measured weekly.

Body weight and body composition, including body fat

Body weight was measured each week until the end of the experiment (15 wk). The body composition was assessed *in vivo* using bioimpedance spectroscopy (BIS) (ImpediVet^{TM}; ImpediMed Ltd., Brisbane, Australia), which is an easy to use, inexpensive and non or minimally invasive analytical technique for measuring the hydration status. The quantity of total body water based on the differential water composition of fat and lean tissues and estimations of the total fat mass (FM), fat-free body mass (FFM), and body mass index (BMI) were determined (Smith and others 2009). The mice were killed after the BIS measurements, the abdominal visceral fat and other organs (liver and kidney) were excised and the wet-weight of each organ was measured.

Administration of D-allulose

In the subchronic studies, the control animals were allowed free access to normal CE2 pellet food and equivalent calories of CE2 containing 2.5% and 5% D-allulose for 15 wk (CLEA Japan, Inc., Tokyo, Japan). D-allulose is basically zero calories. The average daily ingested dose of D-allulose was calculated according to the food intake and body weight. [Estimated dose a day (g / kg / d) = food intake (g / wk) x D-allulose content (g) in one gram pellet (2.5%; 0.025 g or 5%; 0.05 g) / bogy weight (kg) / 7 d].

Assessment of hepatic steatosis using MRI

The MRI data were acquired using a 1.5 Tesla (T) MRmini-SA (DS Pharma Biomedical Co., Ltd, Osaka, Japan) consisting of a solenoid MRI coil with a 40 mm inner dia. The mice were anesthetized with a 1.5 ~ 2.0% isoflurane (160 mL/min, Escain®, MERCK, Kenilworth, N.J., U.S.A.)-oxygen mixture, and the body of each anesthetized mouse was fixed firmly on a polycarbonate holder. The animals were positioned in the MRI coil in such a way that the kidneys were in the approximate isocenter of the coil (and magnet). Five axial slices at the abdominal level and 11 coronal slices were acquired from each mouse. The MRI scans were performed under anesthesia, and the body temperature was measured using a rectal thermocouple and kept constant at 37.5 ± 0.2 °C with a feedback-controlled warm-water blanket (Yamashita Tech System, Tokushima, Japan) connected to a rectal probe (Photon Control Inc. Burnaby BC, Canada) during the MRI scanning. Axial and coronal 3-dimensional Fast Low Angle Shot (FLASH) was used as the basic gradient echo sequence: TR (repetition time) = 50 ms; flip angle = 31.8°, FOV (field of view) = 40 × 80 mm^2; matrix size = 128 × 256 × 64; voxel

size = 0.312×0.312×0.625 mm; NEX (number of excitations) = 4; slice# = 64. In order to assess the degree of hepatic steatosis using MRI, dual echoes corresponding to the chemical shift between water and the dominant fat peak (3.4 ppm) at 1.5T were acquired with FLASH. MR images at TE (echo time) in which the water peak (4.7 ppm) and dominant fat peak (1.3 ppm) were opposed-phase (OP) and MR images at TE in which the two peaks were in-phase (IP) were acquired. Axial 3-dimension FLASH images (TR = 50 ms; TE = 4.4 ms [OP]/6.6 ms [IP]; flip angle = 90°; matrix size = 128×256×128; voxel size = 0.312×0.312×0.312 mm; NEX = 4; FOV = 40 × 80 mm^2; slice# = 128) were also obtained. With respect to quantitative assessment of liver fat with MRI, the fat-signal percentage from the OP and IP signal intensities, S_{OP} and S_{IP}, respectively, was calculated as follows: $(S_{IP} - S_{OP})/2\times S_{IP} \times 100$ (Reeder and others 2011).

Histological analyses using Hematoxylin-Eosin (H&E) and oil-red O staining

For the histological analyses, the mice were deeply anesthetized and euthanized with sodium pentobarbital (50 mg/kg, Sigma-Aldrich Corp., St. Louis, Mo., U.S.A.) and perfused with heparinized 0.1 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4. After perfusion, the livers were removed and postfixed overnight in 4% buffered PFA at 4 °C and then cryoprotected in 30% sucrose. Serial frozen sections (30 μm) were cut on a sliding Cryostat (Leica, CM3050 S, Tokyo, Japan) and mounted onto slides and then dried overnight. The histological studies were performed according to the standard protocols for H&E and oil-red O staining (Lillie and Ashburn 1943). The sections were then covered with a coverslip using Permount™ Mounting Medium, and the liver and adipocyte morphology was evaluated using light microscopy (Olympus U-TB190, Tokyo, Japan).

Culture and differentiation of 3T3-L1 cells

Culture and differentiation of the 3T3-L1 cells were performed based on a previously established method (Phillips and others 1995). 3T3-L1 cells, obtained from ATCC (Rockville, Md., U.S.A.), were cultured in Dulbecco's modified Eagle medium (DMEM) containing 1 mg/mL of D-glucose (Sigma-Aldrich Corp) with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., HyClone, Tokyo, Japan). Over the course of 2 d, confluent 3T3-L1 cells were converted to adipocytes in DMEM containing 1 mg/mL of D-glucose supplemented 1 μM of dexamethasone (Dex), 0.2 mM 3-isobutyl-1-methylxanthine (IBMX), 10 μg/mL of insulin (Ins) and 10% FBS in the presence or absence of 25 mM D-allulose or D-fructose. After 2 d of culture, the cells were kept in the medium containing 10 μg/mL of Ins and 10% FBS, with 25 mM D-allulose or D-fructose. After 4 d of differentiation, the cells began to show visible signs of mature adipocytes, as attested by the appearance of rounded cells with numerous intracellular lipid droplets.

In order to determine the degree of differentiation of 3T3-L1 cells, the glycerol-3-phosphate dehydrogenase (GPDH) activity was measured according to a previous report (Wise and Green 1979) and used as a marker of the adipose activity. 3T3-L1 cells were suspended in extraction buffer (50 mM Tris-HCl, pH 7.5 containing 1 mM EDTA and 1 mM β-mercaptoethanol) and subsequently sonicated to obtain the cell lysate. The amount of NADH consumption depending on dihydroxyacetone phosphate metabolism at room temperature was monitored based on the change in absorbance at 340 nm. One unit of enzyme activity corresponded to the oxidation of 1 nmol of NADH per minute.

Statistical analysis

The differences between the mean values for each group (0%, 2.5%, and 5% D-allulose) were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test. A *P*-value of less than 0.05 was considered to be statistically significant.

Results and Discussion

Subchronic ingestion of D-allulose inhibited body and fat weight, but not food or water intake, in the *ob/ob* mice

Weight gain tended to be lower in the D-allulose-ingested animals than in the controls during the whole study period. After

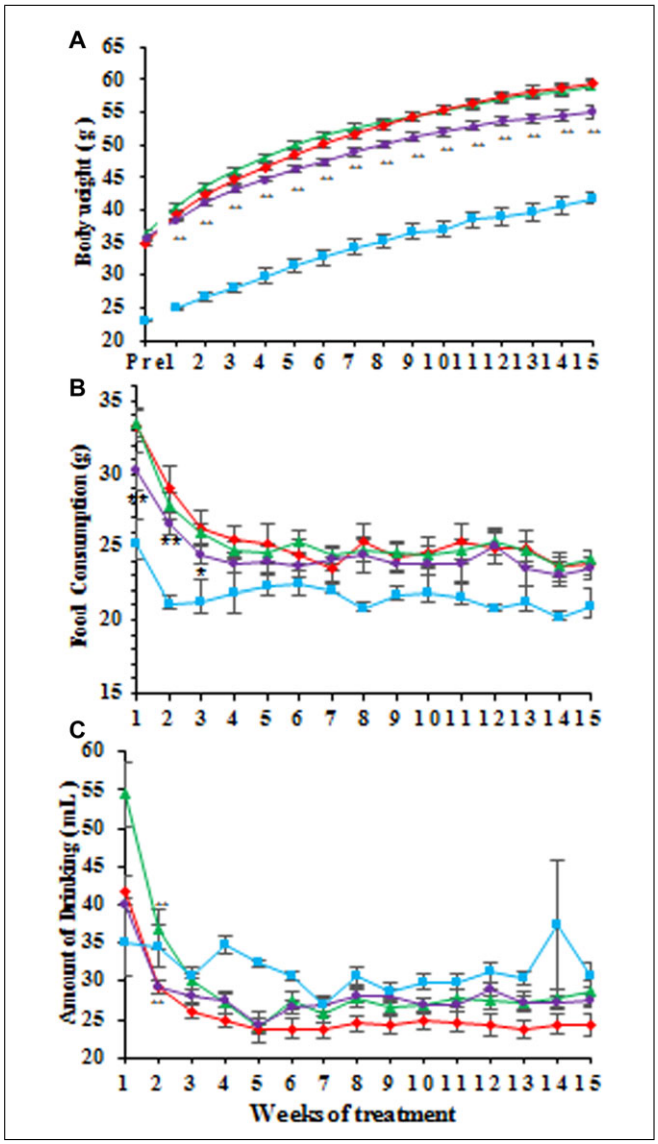


Figure 1–Effects of 2.5% and 5% D-allulose for 15 wk on body weight, food, and water intake in the C57BL/6J and *ob/ob* mice. (A) Body weight, (B) food intake, and (C) water intake. The lines for the *ob/ob* mice show 0% D-allulose (green line, triangle), 2.5% D-allulose (red line, rhombus), and 5% D-allulose (purple line, cycle). The light blue line shows 0% D-allulose in WT (square). The data are expressed as the mean ± standard deviation (n = 14 for all cases). *, $P < 0.05$; **, $P < 0.01$ (compared with 0% D-allulose in *ob/ob* mice).

15 wk of ingestion of 5% D-allulose, the mean body weight was approximately 20% lower in the treated animals than in the control mice ($P < 0.01$) (Figure 1A). Although the food intake was significantly lower in the early period (1 to 3 wk) of ingestion of 5% D-allulose, afterwards amount of food intake showed the trend a decrease (Figure 1B). The average daily ingested-dose of D-allulose was gradually decreased and kept constant after 4 wk (2.5%; 1.5 to 2 g/kg/d, 5%; 3 to 4 g/kg/d). During the 15-wk period, the total calorie intake in the 5% D-allulose ingested mice significantly ($P < 0.01$) decreased by 10% compared to that observed in both the control and 2.5% D-allulose groups (Figure 2A). The trend a decrease in the food intake and the total calorie intake in the 5% D-allulose-ingested *ob/ob* mice is important with respect to the effects of D-allulose, although the meaning of this finding is unclear at this time. Although leptin-deficient *ob/ob* mice have uncontrollable appetites, *ob/ob* mice that ingested D-allulose showed reduced appetites. Therefore, this finding suggests that the reduction of food intake by D-allulose may not be influenced by the leptin pathway. Recently, Nagata and others (2015) showed that a D-allulose diet induced energy expenditure and fat oxidation, but not carbohydrate oxidation, in Sprague–Dawley rats. Their findings indicated that the D-allulose diet decreased lipogenesis and increased energy expenditure, thereby leading to weight management. The organ weight, especially that of the liver, was significantly ($P < 0.05$) decreased by 5% D-allulose (Figure 2B), and the amount of abdominal visceral fat deposition in the D-allulose-ingested mice was 10% ($P < 0.01$) lower than that seen in the control mice (Figure 2D).

Subchronic ingestion of D-allulose decreased body fat in the *ob/ob* mice

In order to determine the FFM and FM of the mouse body composition *in vivo*, we performed BIS using ImpediVet™. The body weights of the 5% D-allulose-ingested *ob/ob* mice were significantly lower than those of the control mice ($P < 0.01$; Figure 3A). The FM and BMI values in the subchronic treated 5% D-allulose *ob/ob* mice were significantly lower than those noted in the control mice ($P < 0.01$) (Figure 3C and D). However, there were no differences in the FFM between the 5% D-allulose-ingested *ob/ob* mice and control mice (Figure 3B). These results suggest that D-allulose decreases the fat content, but not muscle, and thus improves leptin-deficient severe obesity.

MRI findings of the abdominal visceral fat changed following the subchronic ingestion of D-allulose in the *ob/ob* mice

In order to confirm the effect of D-allulose on abdominal visceral fat, an assessment of the abdominal visceral fat area was performed using MRI with the coronal images of FLASH. FLASH MR images exhibiting hyperintense areas in the intraabdominal region indicating abdominal visceral fat; all areas except the kidneys were measured (Figure 4A, b and c; inside red dot lines). The decrease in the hyperintense area was 9.5% following the ingestion of 5% D-allulose in the *ob/ob* mice (0% compared with 5%; 251.0 ± 4.6 mm^2 compared with 215 ± 23.4 mm^2, $P < 0.05$, Figure 4B). The rate of inhibition of hyperintense areas was similar to that for abdominal visceral fat deposition and FM, as described above.

MRI changes associated with hepatic steatosis occurred after the subchronic ingestion of D-allulose in the *ob/ob* mice

In the MRI study, T_1WI signal hyperintense was clearly observed in the livers of the *ob/ob* mice in comparison to the WT mice (Figure 4A, inside yellow solid lines). Therefore, it is

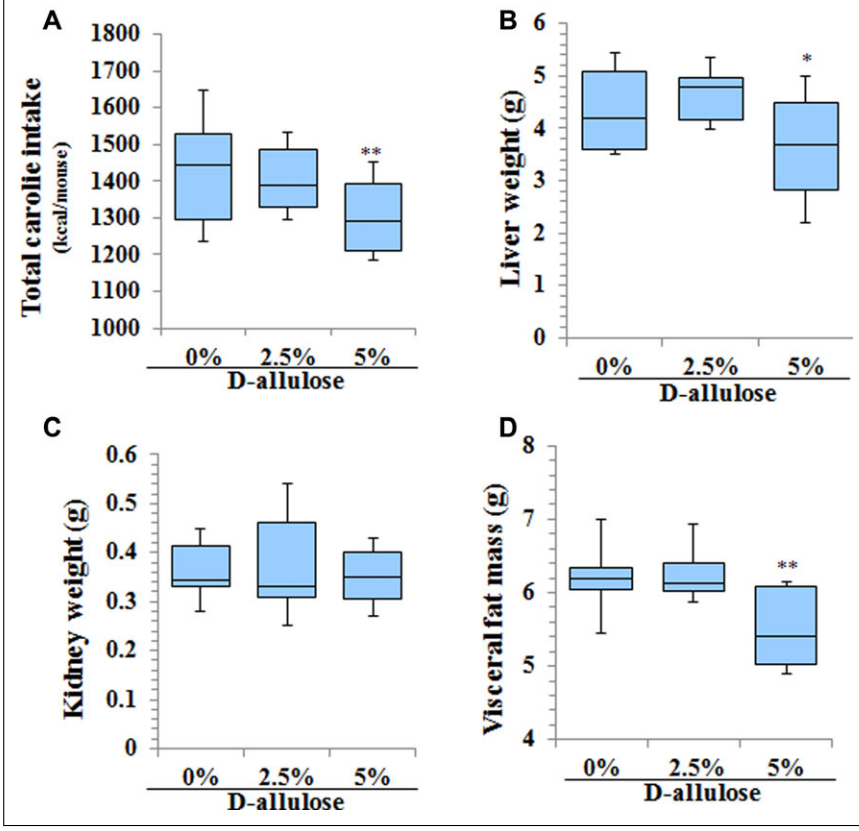


Figure 2–Box plots of the calorie intake and liver, kidney, and abdominal visceral fat weights in the *ob/ob* mice treated with 15wk dietary supplementation of 2.5% and 5% D-allulose. (A) Total calorie intake (kcal/mouse), (B) liver wet weight (g), (C) kidney wet weight (g), and (D) abdominal visceral fat wet weight (g). The box plots show the 25th and 75th percentiles as the upper and lower half of each box along with the 10th and 90th percentiles as upper and lower error bars plus individual outliers (n = 14 for all cases). *, $P < 0.05$; **, $P < 0.01$ (compared with 0% D-allulose in ob/ob mice).

conceivable that the hyperintensity in the liver was due to hepatic steatosis, as fat displays both large longitudinal and transverse regions of magnetization that appear bright on T_1WI (Valls and others 2006). The hyperintensity associated with hepatic steatosis seen in the *ob/ob* mice was inhibited in the 5% D-allulose ingested *ob/ob* mice. In fact, although the color of the liver on gross pathology of the *ob/ob* mice was shell pink, the liver color changed to rose red after 15 wk of 5% D-allulose ingestion (Figure 4A, h and i; inside yellow dot lines).

In terms of the degree of hepatic steatosis on MRI, gradient-dual echo IP and OP sequences have been utilized to assess hepatic MRI findings in humans (Fishbein and others 1997; Rinella and others 2003). In the current study, attempted to observe hepatic steatosis in the *ob/ob* mice according to the dual echo protocol at 1.5T. As a result, hyperintense and hypointense areas in IP and OP, respectively, were noted in the livers of the *ob/ob* mice (Figure 5A). The difference in the dual phase in the liver indicates hepatic steatosis (Fishbein and others 1997; Rinella and others 2003). In the subchronic D-allulose-ingested *ob/ob* mice, the difference in the signal intensity between IP and OP was less (Figure 5A). Meanwhile, the fat signal percentage in the livers of the *ob/ob* mice was significantly inhibited by 30% in the 5% D-allulose-ingested *ob/ob* group (0% compared with 5% D-allulose; 30.0 ± 2.9% compared with 20.9 ± 4.1%, $P < 0.01$, Figure 5B). Therefore, the MRI evidence indicated that the subchronic ingestion of 5% D-allulose improves hepatic steatosis in *ob/ob* mice.

Histological changes in the liver following the subchronic ingestion of D-allulose in the *ob/ob* mice

In order to confirm the improvement in hepatic steatosis achieved with D-allulose in *ob/ob* mice, histological analyses were performed using H&E and oil-red O staining of frozen liver sections. Consequently, fat deposition produced a severely damaged liver histology presenting as remarkable ballooning degeneration in the nontreated *ob/ob* mice (Figure. 6B, E, H, and K). However, the ballooning degeneration and hepatic steatosis improved after the subchronic ingestion of D-allulose (Figure 6C, F, I, and L). Furthermore, in the *ob/ob* mice, the volume of adipocytes in the adipose tissues evidently increased (Figure 6H, I, K, and L), and the weight of the liver following the ingestion of 5% D-allulose was significantly smaller than that observed in the control livers ($P < 0.05$, Figure 2B). Therefore, the liver histological evaluations clearly showed that the 15 wk of ingestion of 5% D-allulose improved the hepatic steatosis in *ob/ob* mice.

D-allulose inhibited the differentiation of 3T3-L1 cells *in vitro*

Adipocytes are the major cell types in adipose tissues, and excessive growth and differentiation of adipocytes are critical factors in the development of obesity. It has been previously suggested that D-allulose may inhibit adipose tissue differentiation. Therefore, in order to investigate whether D-allulose influences the inhibition of adipocyte differentiation, murine 3T3-L1 cells (preadipocytes)

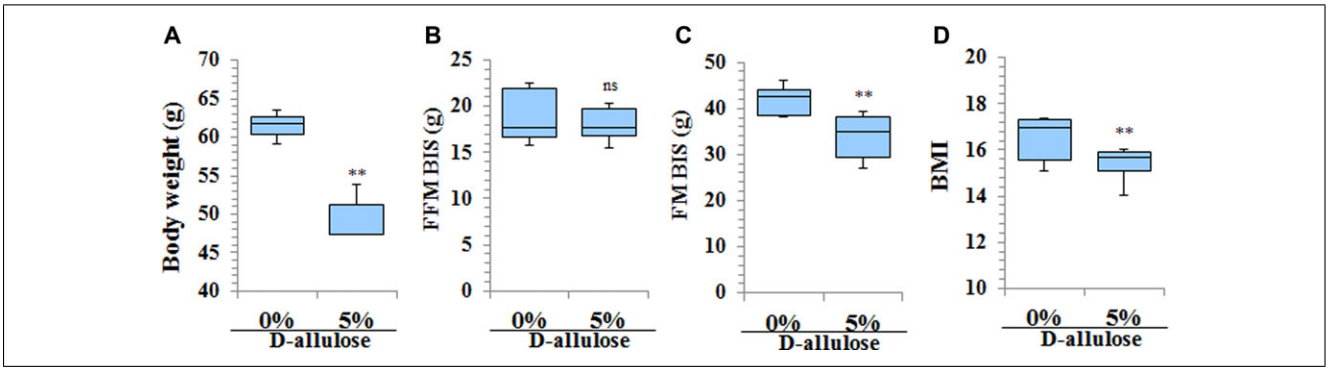
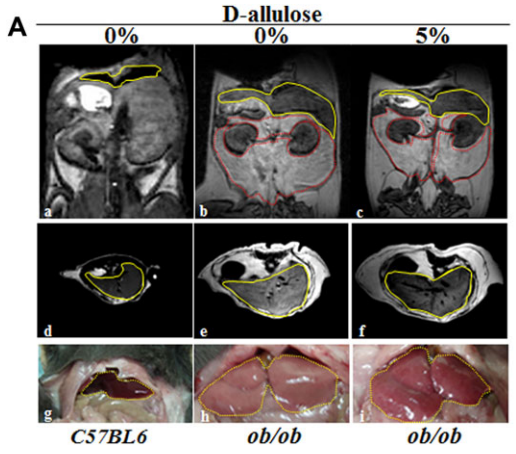


Figure 3–Box plots for the BW, FFM, FM, and BMI values in the *ob/ob* mice treated with 15wk dietary supplementation of 5% D-allulose. (A) Body weight (g), (B) FFM BIS (g), (C) FM BIS (g), and (D) BMI after 15 wk of ingestion of 5% D-allulose. The box plots show the 25th and 75th percentiles as the upper and lower half of each box along with the 10th and 90th percentiles as upper and lower error bars plus individual outliers (n = 14 for all cases). ns; not significant, **, $P < 0.01$ (compared with 0% D-allulose).



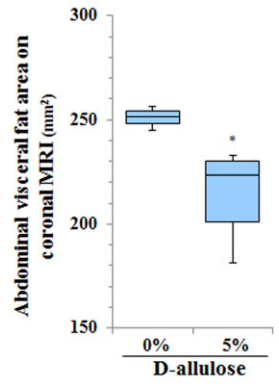


Figure 4–MRI images and gross pathology of abdominal visceral fat deposits and hepatic steatosis in the *ob/ob* mice treated with 15wk dietary supplementation of 5% D-allulose. (A) Representative coronal (a to c) and axial (d to f) MR images and photographs of gross pathology (g to i). (B) The box plots of the abdominal visceral fat area inside the red line (mm^2) on coronal MRI show the 25th and 75th percentiles as the upper and lower half of each box along with the 10th and 90th percentiles as upper and lower error bars plus individual outliers (n = 6 for all cases). *, $P < 0.05$ (compared with 0% D-allulose).

were used in an *in vitro* study. 3T3-L1 cells show fibroblast-like morphology without stimulation, but undergo adipocyte differentiation upon induction by three factors, IBMX, Dex, and Ins, which is characterized by the acquisition of lipid storage droplets and the expression of fat cell markers, such as GDPH. The differentiated 3T3-L1 adipocytes mimic the adipocytes isolated from adipose tissues (Green and Kehinde 1975) and thus are widely used in the field of adipocyte differentiation as well as lipid metabolism (Poulos and others 2010). The addition of Dex, IBMX, and Ins to the culture of 3T3-L1 cells elicited a morphological change toward a round shape (Figure 7A, a to d), an increment in the number of oil-red O stained cells (Figure 7A, e to h) and an increase in the GPDH activity (Figure 7B), clearly indicating the differentiation of 3T3-L1 cells to adipocytes. The application of 25 mM D-allulose to the culture effectively suppressed the differentiation of 3T3-L1 cells to adipocytes (Figure 7A, a to d) as well as increased the GPDH activity (Figure 7B), accompanied by adipose differentiation 4 d after the addition of Dex, IBMX and Ins. In the differentiated 3T3-L1 adipocytes, a decrease in lipid droplets following the application of 25 mM D-allulose was also observed on oil-red O staining (Figure 7A, d and h) on day 5. Treatment of the 3T3-L1 cells with 25 mM D-fructose caused no changes in the cellular morphology, GPDH activity or amount of lipid droplets compared with the control cells (Figure 7). These data indicate that D-allulose, but not D-fructose, suppresses the differentiation of 3T3-L1 cells to adipocytes and thus suggest the inhibition of the differentiation of preadipocytes to adipocytes and the accumulation of fat in the adipocytes, which may be a potential target for treating obesity.

This study provides evidence that dietary supplementation with D-allulose prevents against the development of hepatic steatosis in *ob/ob* mice, without exercise therapy or dietary restriction. Furthermore, the histological and MRI evidence clearly showed that 15 wk of the ingestion of 5% D-allulose reduced hepatic

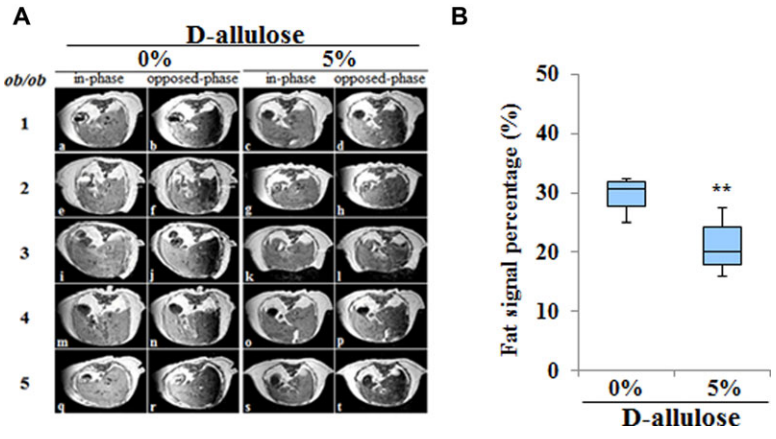


Figure 5–Axial MR images of a liver illustrating in-phase and opposed-phase images and the fat signal percentage in the *ob/ob* mice treated with 15wk dietary supplementation of 5% D-allulose.
(A) Axial in- and opposed-phase MR images of 5 animals (1 to 5) presenting in-phase (a, e, i, m) to opposed-phase (b, f, j, n) images in the 0% D-allulose mice and in-phase (c, g, k, o) to opposed-phase (d, h, l, p) images in the 5% D-allulose mice. (B) The box plots of the fat signal percentage (%) in the liver on axial MR images show the 25th and 75th percentiles as the upper and lower half of each box along with the 10th and 90th percentiles as upper and lower error bars plus individual outliers (n = 5 for all cases). **, $P < 0.01$ (compared with 0% D-allulose).

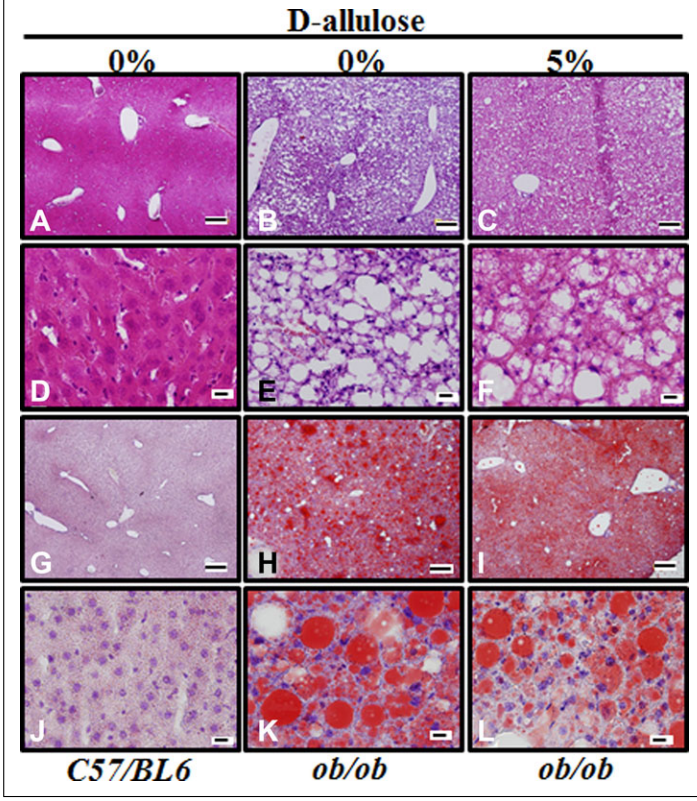


Figure 6–Representative photomicrographs of liver sections stained with H&E and oil-red O staining in the *ob/ob* mice treated with 15wk dietary supplementation of 5% D-allulose. H&E staining of livers from the 0% D-allulose-ingested C57BL/6J (A, D), 0% D-allulose- (B, E), and 5% D-allulose-ingested *ob/ob* mice (C, F). Oil-red O staining of the livers from the 0% D-allulose-ingested C57BL/6J (G, J), 0% D-allulose (H, K), and 5% D-allulose-ingested *ob/ob* mice (I, L). The scale bars indicate 200 μm at low magnification (A, B, C, G, H, I) and 20 μm at high magnification (D, E, F, K, J, L).

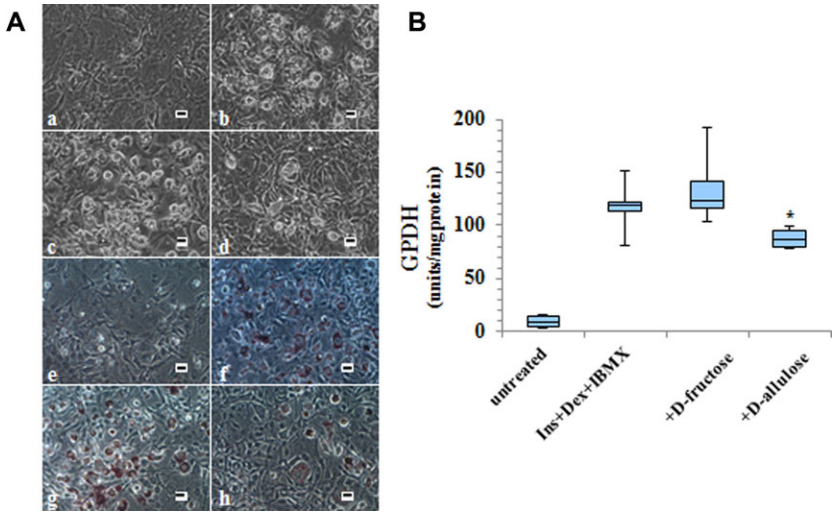


Figure 7–Effects of D-allulose treatment on the Dex-, IBMX- and Ins-induced differentiation and lipid accretion in differentiated 3T3-L1 adipocytes. (A) Representative photomicrographs show undifferentiated 3T3-L1 cells (a, e) and morphological differentiation in the absence (b) or presence of 25 mM D-fructose (c), and 25 mM D-allulose (d). Lipid accumulation stained with oil-red O in differentiated 3T3-L1 adipocytes in the absence (f) or presence of 25 mM D-fructose (g) and 25 mM D-allulose (h). The scale bars indicate 50 μm. (B) The box plots of the GPDH activity used as an adipose marker show the 25th and 75th percentiles as the upper and lower half of each box along with the 10th and 90th percentiles as upper and lower error bars plus individual outliers (n = 4 for all cases). *, $P < 0.05$ (compared with 0% D-allulose).

steatosis. Previous reports have suggested that D-allulose inhibits the activities of several lipogenic enzymes in the liver, resulting in lower abdominal fat accumulation in rats (Matsuo and others 2001). We suggest that the reduction of abdominal and liver fat accumulation in *ob/ob* mice may be the result of prevention of differentiation in adipocytes induced by D-allulose. Although D-allulose is an epimer of D-fructose isomerized at the C-3 position (Matsuo and others 2001; Baek and others 2010), the sweetness of D-allulose is approximately 70% of that of D-fructose and it has zero calories (Oshima and others 2006). We recently showed that D-allulose serves as a unique metabolic regulator in growing type 2 diabetes OLETF rats via the maintenance of blood glucose and prevention of abdominal fat deposition (Hossain and others 2011). In *ob/ob* mice, the oral administration of 5% D-allulose solution for 10 wk results in a decline in the $AUC_{glucose}$ on oral glucose tolerance test (OGTT), but not the fasting blood glucose levels (data not shown). In WT mice, however, postprandial hyperglycemia and the $AUC_{glucose}$ on OGTT are not influenced by 5% D-allulose (data not shown). These results indicate the supplemental benefits of D-allulose, especially on obesity, but not under normal conditions.

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease (Sass and others 2005). Previous studies have shown that NAFLD is strongly associated with obesity, (Ludwig and others 1980; Powell and others 1990), insulin resistance (Powell and others 1990; Marchesini and others 1999; Sanyal 2002) and type II (noninsulin dependent) diabetes mellitus (Powell and others 1990; Sanyal 2002). Weight loss and reducing hepatic steatosis are particularly important for the primary treatment of NAFLD. *ob/ob* mice are genetically leptin-deficient and spontaneously become obese, and obesity is considered to be a good model of NAFLD (Anstee and Goldin 2006). Therefore, the present results provide important findings regarding the relationship between D-allulose and improvements in NAFLD in *ob/ob* mice.

Conclusion

We consider that the effect of D-allulose may be primarily mediated by affecting body weight and reducing hepatic steatosis in the severe obesity model without exercise therapy or dietary restriction. The present findings indicated that the rare sugar D-allulose maintains the body weight and prevents abdominal and hepatic fat accumulation under severe conditions of obesity in mice and is thus expected to be approved for commercial use as a substitute for natural sugar in foodstuffs with the goal of controlling obesity and obesity-related diseases, such as hepatic steatosis and diabetes. However, further prospective evaluations are necessary to elucidate the mechanisms of action underlying repeated D-allulose supplemental treatment for reducing obesity, including weight loss and fatty liver diseases.

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Author Contributions

K. Itoh designed and conducted the study, interpreted the results and drafted the manuscript. S. Mizuno collected and analyzed the histological data. S. Hama collected and analyzed the MRI data. W. Oshima and M. Kawamata collected and analyzed the blood glucose data. M. Tokuda and A. Hossain analyzed the body composition data and interpreted the results. Y. Ishihara collected and analyzed the *in vitro* (3T3-L1 cell culture) data and interpreted the results.

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