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Rare sugar D-psicose protects pancreas β-islets and thus improves insulin resistance in OLETF rats

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ABSTRACT

Rare sugar D-psicose has cropped up as a non-toxic and effective compound to protect and preserve pancreatic β-islets in the growing type 2 diabetes mellitus (T2DM) rats through the regulation of glucose and fat metabolism. The present study was undertaken to examine the effect of rare sugar D-psicose on the protection of pancreatic β-islets using Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a T2DM model. Treated rats were fed with 5% D-psicose or 5% D-glucose supplemented drinking water, and only water in the control for 13 weeks. A non-diabetic Long-Evans Tokushima Otsuka (LETO), fed with water served as a counter control of OLETF. D-Psicose significantly attenuated progressive β-islet fibrosis and preserved islets, evaluated by hematoxylin–eosin staining, Masson's trichrome staining and immunostainings of insulin and α-smooth muscle actin (SMA). D-Psicose significantly reduced increase in body weight and abdominal fat deposition. Oral glucose tolerance test (OGTT) showed reduced blood glucose levels suggesting the improvement of insulin resistance. All these data suggests that D-psicose protected and preserved pancreatic β-islets through the maintenance of hyperglycemia and by the prevention of fat accumulation in OLETF rats.

1. Introduction

Prevalence of global obesity has been increasing with alarming health problems day by day. Obesity is characterized by excess accumulation of fat and is associated with multiple complications, including T2DM [1] which also becomes a health problem and is thought to typically develop after age 40, but it is now increasingly seen in younger ages as obesity increases and physical activity decreases.

In T2DM the body either does not produce enough insulin or there is insulin resistance resulting in the failure of sugar movement into the cells [2]. Initially, pancreatic β-islets produce more insulin to overcome the hyperglycaemic state resulting in islet hypertrophy that gradually proceeds to β-cell failure [3] and progressive failure leads to glucose intolerance followed by T2DM. Studies with rodents showed that decrease in β-cell mass play important roles in the pathogenesis of human T2DM [2]. If remains untreated, the consequences of T2DM can be life-threatening. Although there is no cure for T2DM, but it is manageable or even preventable, starting by eating healthy foods, exercising and maintaining body weight. Therefore, it is important to seek effective therapeutic interventions for the prevention and treatment of diabetes and its complications. Under these circumstances, the use of alternative medicines has increasingly become the focus of attention. And thus we introduce D-psicose, a non-toxic zero-calorie rare sweet monosaccharide as an effective agent against growing obesity and T2DM.

D-Psicose, an epimer of D-fructose isomerized at C-3 position, is a rare ketohexose, originates from wheat, itea plants, processed cane and beet molasses [4], also present in commercial complexes of D-glucose and D-fructose in small quantities [4]. Due to rarity the biological functions have not been explored but innovating unique methods of production through Izumoring [5,6], enables a number of investigations [4] and expected to be approved for commercial use as a substitute of sugar in foodstuffs aiming at maintaining the physiological levels of blood sugar, preventing excess fat deposition and thus controlling obesity.

D-Psicose has attracted much attention for its promising anti-hyperglycemic and anti-lipidemic effects, shown experimentally in normal rats and clinically in maltodextrin-stimulated glucose tolerance test in healthy humans [7,8]. And most recently we have shown that D-psicose served as a unique metabolic regulator in the

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growing type T2DM OLETF rats through the maintenance of blood glucose and prevention of abdominal fat deposition [9]. These results have proposed that D-psicose could be a suitable candidate for antidiabetic agent even as food ingredients. However, in the present study, we examined the effect of D-psicose in the prevention of pancreas β-islets from hyperglycemia-induced islet fibrosis in T2DM OLETF rats.

2. Materials and methods

2.1. Animals

Male OLETF and LETO rats were housed and maintained with controlled temperature (25 °C) and lighting (12 h light/dark cycle) and handled in compliance with the Guide for Experimental Animal Research. After 1-week adaptation OLETF rats were divided into 3 groups (n = 15 each): control was given drinking water, psicose group 5% D-psicose and glucose group 5% D-glucose, in drinking water and control LETO was given only drinking water.

2.2. Food intake

Animals were allowed free access to water and normal CE2 pellet foods. Food intake for 3 consecutive days each week was measured to calculate the average of g/100 g body weight consumption and drink quantity was also measured.

2.3. Measurements of obesity

2.3.1. Physical appearance, body weight and abdominal obesity

Change of physical appearance or behavior was observed every day. Body weight was measured every week till sacrifice. During sacrifice, after blood collection rats were perfused with normal saline followed by 4% paraformaldehyde, total abdominal fat and other organs were dissected, weighed and preserved accordingly for further analysis.

2.3.2. Body composition including body fat

For *in vivo* body composition assessment we used bioimpedance spectroscopy (ImpediVet, ImpediMed Ltd., Brisbane, Australia), which is an easy to use, inexpensive and non- or minimally invasive analytical technique for the measurement of hydration status. Quantity of total body water (TBW), and based on differential water composition of fat and lean tissues, an estimation of the total fat mass (FM) fat-free body mass (FFM) and body mass index (BMI) was determined [10].

2.4. Characterization of glucose metabolism

2.4.1. Periodical blood glucose levels and oral glucose tolerance test (OGTT)

Fasting blood glucose was measured periodically using a freestyle glucose meter (YSI 2300-STAT) by simple needle-prick at rat tail tip. For OGTT, each animal was fed 2.0 g/kg of 50% glucose solution and blood was collected at 30, 60, 90, and 120 min after glucose load. The area under curve for glucose ($AUC_{glucose}$) was calculated using the trapezoid rule for glucose data from 0 to 120 min.

2.4.2. Degree of insulin resistance

Homeostasis model assessment (HOMA) was calculated according to the formula, for insulin resistance (HOMA-IR): insulin (μ-units/ml) × glucose (mmol/l)/22.5, for β-cell function (HOMA-b): insulin (μ-units/ml) × 20/glucose (mmol/l) − 3.5 [11]. For insulin sensitivity (HOMA-%S) we used a calculator from internet (HOMA2 calculator, University of Oxford, 2004).

2.5. Inflammatory profile

2.5.1. Hematoxylin–eosin (HE) staining

Formalin-fixed, paraffin-embedded pancreas tissue and epididymal adipose tissue were stained with HE. Adipocyte morphology was evaluated by light microscopy and cell number was counted using IMageJ software. Degree of pancreas tissue damage was assessed by evaluating changes in the islets; shape of islets (architecture), vacuolization, fibrosis and intra-islet congestion and a semiquantitative rating ranging from 0 (intact) to 3 (severe) was assigned to each component [12].

2.5.2. Immunostaining for β-cell mass and fibrosis

Pancreas tissue was embedded in optimal cutting temperature (OCT) compound and stained with anti-insulin antibody (Funakoshi, Japan) for the measurement of β-cell mass and the number was counted using ImageJ software. Cell mass was calculated by multiplying the relative percentage of β-cells by the total pancreatic weight and the percentage was calculated from insulin-positive staining area of each slide [13]. To investigate the degree of islet fibrosis sections were stained with Masson's trichrome and the percentage of fibrotic blue-stained area was calculated by ImageJ software. Sections were also stained using a polyclonal rabbit anti-human mouse smooth muscle α-actin (α-SMA, Sigma St. Louis, MO), and counted similarly.

2.5.3. Serum levels of adipocytokines

Serum levels of leptin and adiponectin were measured by rat-specific Quantikine ELISA kits (R&D systems, Minneapolis, USA). All assays were performed in duplicate according to the manufacturer's recommendations.

2.6. Statistical analysis

Data are expressed as means ± SD. Statistical comparison of the means among the groups was made using one-way ANOVA. Differences between groups were analyzed by the post hoc Hoechberg's for equal and Games–Howell for unequal variances using SPSS software (version 17.0, SPSS, Chicago, IL). A p value of <0.05 was considered significant.

3. Results

3.1. Effect of D-psicose on physical appearance, body weight and food intake

Physical appearance of control OLETF rats was bulky than other rats. No behavioral changes were observed. Weight gain tended to be lower in the D-psicose-treated animals than in controls during the whole study period. During sacrifice mean body weight was 114.84 and 109.34 g lower in D-psicose group compared with control ($p < 0.01$) and D-glucose ($p < 0.01$) groups, (Fig. 1A). Food intake by 100 g body weight was non-significantly lower for initial few weeks of treatment in D-psicose group but for the further period there was no difference (Fig. 1B).

3.2. Effect of D-psicose on obesity

3.2.1. Abdominal adiposity

Total amount of fat deposition (epididymal + perirenal) was significantly lower in D-psicose-treated rats than other OLETF rats (25.45 g in the control group and 24.34 g in the D-glucose group vs 13.26 g, ($p < 0.001$) in D-psicose group) (Fig. 1C). Control OLETF rats accumulated large-size droplets of lipid evaluated by the microscopic size of adipocytes (Fig. 2B) where

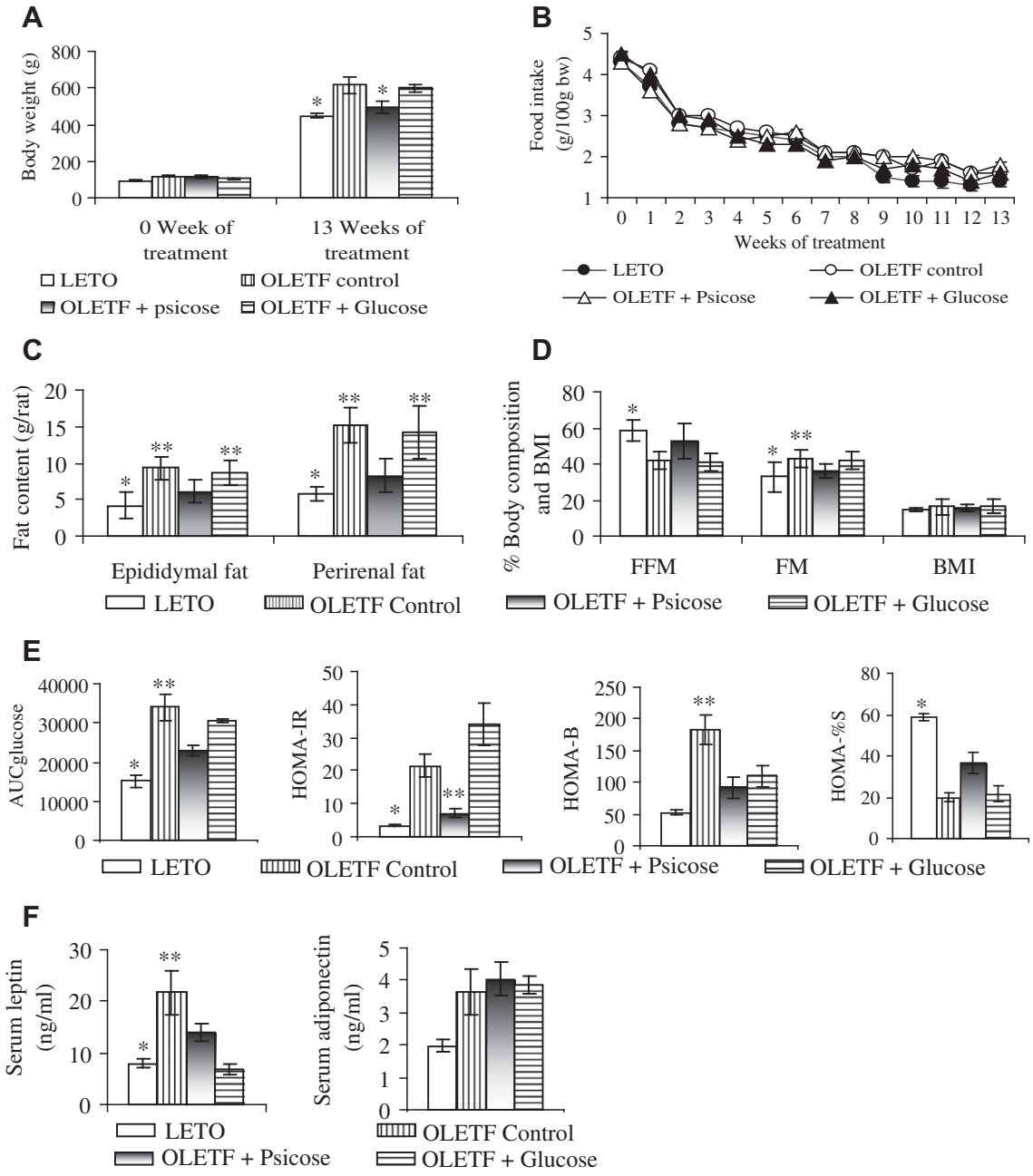

Fig. 1. Effect of D-psicose on body weight, food intake, body composition, adipocytokine and insulin resistance in OLETF and LETO rats. Body weight and food intake (A and B), abdominal fat and body composition (C and D), insulin resistance status; area under curve glucose ($AUC_{glucose}$), homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis assessment of β-cell function (HOMA-b) and homeostasis assessment of insulin resistance (HOMA %S) (E), serum leptin and adiponectin (F). Results are expressed as means ± SD, $n = 10$ (LETO), 15 (OLETF per group). $^{*}p < 0.001$ vs OLETF; $^{**}p < 0.01$ vs D-psicose.

the psicose-treated OLETF rats showed smaller lipid droplets (Fig. 2C) and due to the presence of small-sized droplets the number of adipocytes was significantly increased in D-psicose group (Fig. 2E) compared with control group, reflecting aggregates of small fat cells.

3.2.2. Percent body composition and BMI

Fat mass (FM) was significantly lower in D-psicose rats than both control and D-glucose rats (D-psicose vs control, 36.01 ± 4.17% vs 43.11 ± 4.95%, $p < 0.05$; D-psicose vs D-glucose, 36.01 ± 4.17% vs 41.70 ± 4.75%, $p < 0.05$) (Fig. 1D). The corresponding mean fat free body mass (FFM) percentages were non-significantly higher in the LETO and D-psicose groups than control and D-glucose groups. There was no significant difference of BMI among the groups.

3.3. Effect of D-psicose on glucose metabolism

3.3.1. Periodical blood glucose concentration

Periodical blood glucose levels were higher in the control and D-glucose groups. On sacrifice day, the level was significantly lower in D-psicose group than other OLETF groups (psicose vs control, 108.0 ± 12.5 mg/dl vs 135.0 ± 26.8 mg/dl, $p < 0.05$; psicose vs glucose, 108.0 ± 12.5 mg/dl vs 226.0 ± 50.0 mg/dl, $p < 0.05$; psicose vs LETO, 108.0 ± 12.5 mg/dl vs 94.0 ± 6.0 mg/dl). All the rats in control and D-glucose groups had a glucose concentration greater

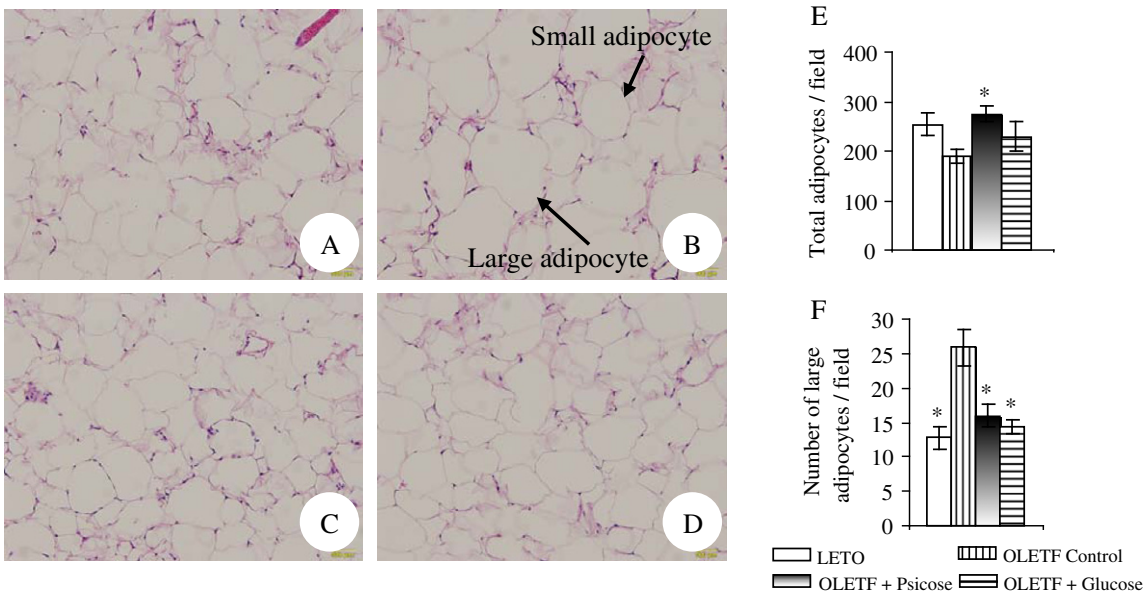

Fig. 2. Effect of D-psicose on epididymal white adipose tissue morphology in OLETF and LETO rats; (A) LETO, (B) OLETF control, (C) OLETF psicose and (D) OLETF glucose. HE-stained paraffin-embedded sections (A–D) and number of adipocytes per field (E–F). Values are mean ± SE, $^{*}p < 0.01$ vs OLETF control.

than 120 mg/dl where only one rat in D-psicose group had (individual data not shown).

3.3.2. OGTT glucose concentration and HOMA

Blood glucose values were non-significantly lower at 30 min and significantly at both 60 and 90 min in D-psicose group than others, (psicose vs control, 240.20 ± 7.30 mg/dl vs 423.14 ± 88.05 mg/dl; psicose vs glucose, 240.20 ± 7.30 mg/dl vs 411.57 ± 48.06 mg/dl) at 60 min, and (psicose vs control, 150.00 ± 46.69 mg/dl vs 310.33 ± 69.04 mg/dl); psiocse vs glucose, 150.00 ± 46.67 mg/dl vs 244.00 ± 32.05 mg/dl) at 90 min. Glucose values in the non-diabetic LETO rats were significantly lower than all OLETF groups in all the time points (data not shown). The mean value of $AUC_{glucose}$ was significantly lower ($p < 0.001$) in psicose group than OLETF control and glucose groups (Fig. 1E).

The HOMA-IR and HOMA-b indexes were also significantly higher in OLETF control and glucose groups, indicating the presence of IR (insulin resistance) state and β-cells attempt to cope with increased insulin demand in these rats. All these high levels decreased significantly in D-psicose group. Consequently HOMA-%S (insulin sensitivity) was increased significantly in D-psicose group (Fig. 1E).

3.4. Effect of D-psicose on pancreas status and adipose tissue

3.4.1. Pancreas morphology with HE staining

Striking differences were observed in pancreas islet morphology among the groups. Islets of OLETF control rats were large, disorganized with irregular shape, separated into clusters and shown expanded into the adjacent exocrine tissues. Extensive fibrosis and fatty degeneration were also marked in the hypertrophied islets (Fig. 3B arrows). The above-mentioned features were prominent in the control rats and less prominent in the D-glucose-fed rats, while almost absent with relatively well-preserved islets in the D-psicose-fed rats, where the islets were organized with small and round or oval regular shapes, minimum fibrosis, and less fatty deposition (Fig. 3C). The islets of non-diabetic LETO rats found normal. In semi-quantitative rating of tissue injury, vacuolization and fibrosis were moderate to severe in most of the rats in OLETF control whereas mild in most rats in D-psicose group (Fig. 3E). Intra-islet congestion was moderate in most control rats whereas no congestion in D-psicose rats.

3.4.2. Insulin immunostaining

Intense brown, dense insulin-positive staining was seen most frequently in D-psicose-fed rats relative to the control rats (Fig. 4A–C). The distribution of endocrine cells in each islet was within normal pattern in D-psicose rats, whereas, it showed attenuated and variable pattern of staining with indistinct and less insulin-stained β-cells, suggestive of progressive β-cell loss in OLETF control (Fig. 4B). Quantification analysis revealed non-significantly higher β-cell mass in the psicose-treated rats than control, (D-psicose vs control, 55.70 ± 12.9 mg/dl vs 47.15 ± 14.7 mg/dl) (Fig. 4M), which may explain relatively reduced size of pancreas in diabetic OLETF rats. However, treatment with D-psicose restored β-cell mass compared with untreated OLETF controls.

3.4.3. a-SMA immunostaining

Multiple intra-islet immunostaining was frequent in larger and degenerated islets of the control rats (Fig. 4F), suggestive of proliferative microvessels. Inversely, weak-stained α-SMA was observed only in the peri-islet area, not in the islets, suggesting the absence of smooth muscle formation in D-psicose-treated rats (Fig. 4G). Semi-quantitative rating also revealed significant higher %α-SMA-stained area per islet in the control group than all other groups (Fig. 4N).

3.4.4. Islet fibrosis with Masson's trichrome staining

Degree of islet fibrosis was assessed using Masson's trichrome stain (blue staining) to investigate the altered morphology and loss of β-cells, a common feature of rat models of diabetes with progressive β-cell loss. Islets of control rats were enlarged and disrupted at boundaries and showed wide area of advanced degree of fibrosis (Fig. 4J, arrows). In the enlarged islets, there were various extents of connective tissue proliferation which widely separated endocrine cells, giving the appearance of multilobules or clusters. Similar features were also shown by D-glucose-treated rats (Fig. 4L). In contrast, treatment with D-psicose presented significant diminution in fibrosis intensity (Fig. 4K). Semi-quantitative rating

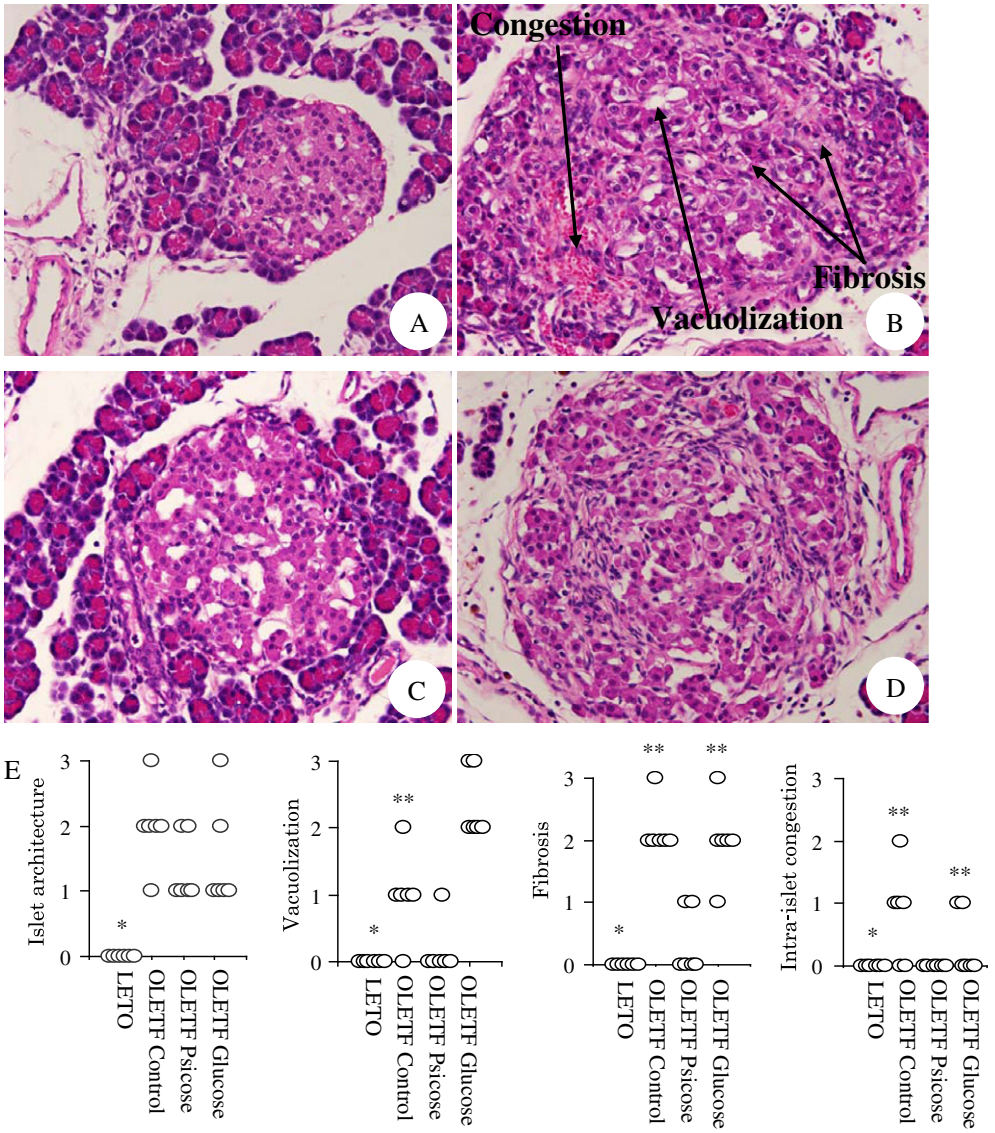

Fig. 3. Effect of D-psicose on the morphological changes of pancreas tissue in OLETF and LETO rats; (A) LETO, (B) OLETF control, (C) OLETF psicose and (D) OLETF glucose. HE-stained paraffin-embedded sections (A–D) and semiquantitative rating of the shape of islets (architecture), vacuolization, fibrosis and intra-islet congestion (E) ranging from 0 (intact) to 3 (severe) was assigned to each component. Values are mean ± SE, $^{*}p < 0.001$ vs OLETF; $^{**}p < 0.01$ vs D-psicose.

also showed significant higher %fibrosis area/islet in both control and D-glucose groups (Fig. 4O).

4. Serum levels of adipocytokines

As shown in Fig. 1F, serum leptin level decreased significantly in the D-psicose group compared with the control group at the end of experimental period. Serum adiponectin level was non-significantly higher in D-psicose group compared with control.

5. Discussion

Rare sugar D-psicose protected hyperglycaemic overloaded pancreas in T2DM OLETF rats through preserving β-islets and thus maintained blood glucose levels. β-Cell dysfunction and insulin resistance are the two predominant and characteristic features of T2DM. In this event, insulin resistance is usually diagnosed before β-cell dysfunction is detected [14]. Insulin resistance subjects maintain normal glucose tolerance by adaptive hypersecretion of insulin and in certain individuals, compensatory mechanisms become inadequate with time and overt hyperglycemia with clinical diabetes supervenes [15]. The resulting β-cell failure of the overworked pancreas that compensate for insulin resistance and the action of D-psicose against this failure are thus key to our understanding of the present study.

This study showed that D-psicose maintained blood glucose levels in T2DM within normal physiological range throughout the entire experimental period. D-Psicose also prevented excess fat accumulation in the abdomen. Furthermore, D-psicose prevented pancreatic β-islets from hyperglycemia-induced β-cell loss and islet fibrosis, which was our main concern in the present study, since enumerable studies have already confirmed the glucose maintenance effect of D-psicose both experimentally [16,17] and clinically [8,18]. As the mechanisms mentioned D-psicose reduced or delayed glucose absorption in the intestine by the retardation of the digestive enzyme, α-glucosidase [17]. And most recently we have shown that D-psicose maintained blood glucose levels in T2DM OLETF rats through the translocation of glucokinase (GK) in the hepatocytes [9]. The presence of GK in the hepatocyte cytoplasm is obligatory

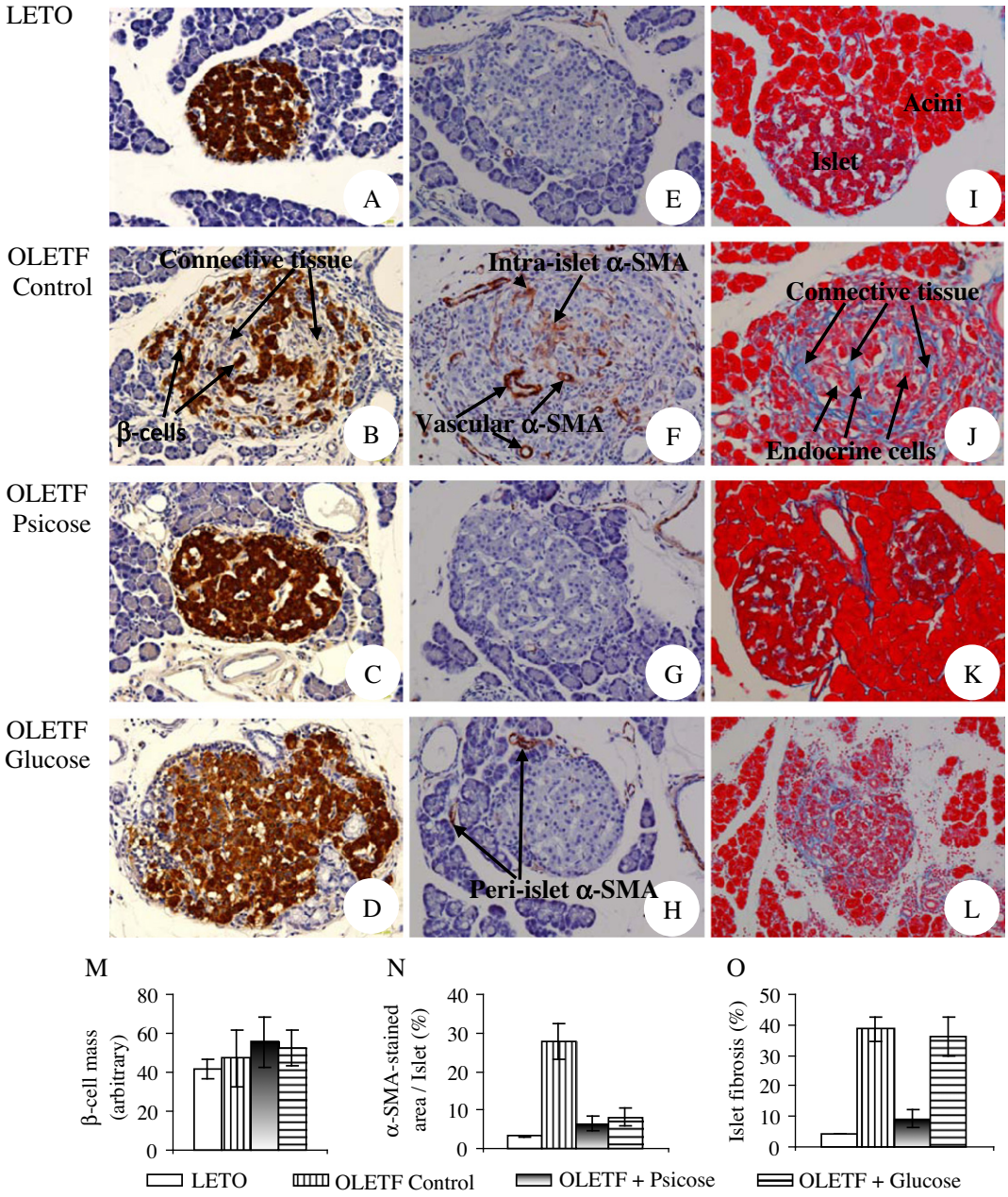

Fig. 4. Immunostaining for insulin (A–D) and α-SMA (E–H), and Masson's trichrome staining (I–L, collagen fibers are shown in blue) of pancreas of LETO and OLETF rats. β-Cell mass, %α-SMA-stained area and %islet fibrosis were shown in M–O. Fibrosis in the insulin-stained islets and Masson's-stained islets was shown by arrows. Intense brown-colored α-SMA staining was observed in ring-shaped vessels and in the peri-islet areas in the enlarged and disorganized pancreatic islets of OLETF control rats (F). Values are mean ± SE, $^{*}p < 0.001$ vs OLETF; $^{**}p < 0.01$ vs D-psicose. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for glucose transportation [19] and it has been shown that GK expression in the liver is progressively reduced with the development of hyperglycemia [9]. In this connection, we performed OGTT since blood glucose level after OGTT reveals the actual status of the pancreas and thus OGTT remains one of the main risk factors parameter for the progression of T2DM. Treatment with D-psicose achieved the significant improvement in OGTT (Fig. 1E).

Another important measure of whole body resistance is HOMA, calculated from glucose and insulin data. Higher indexes of HOMA-IR and HOMA-b in OLETF control rats confirmed insulin resistance state and feeding D-psicose maintained these indexes, indicating that IR was not developed in treated animals and thus prevented the consequent β-cell function overload. β-Cell function cannot be interpreted in the absence of measuring insulin sensitivity, and therefore HOMA-%S should always be reported alongside HOMA-b where %S is a function of glucose metabolism driven by the action of insulin [11].

Here, till this point of our study pancreas is the main target organ where we observed striking morphological differences between D-psicose-treated and non-treated rats. Control islets were hypertrophic and more irregular in shape with disorganized cell architecture and progressive β-cell loss, inversely, islets in D-psicose rats showed small and oval with normal cell architecture (Fig. 3C). These findings were consistent with the study which mentioned the progressive histopathological changes including selective loss of β-cells and fibrosis with the development of

T2DM [20]. So, the most obvious mechanism to explain pancreatic decompensation is a progressive loss of β-cell mass, which is hastened by islet fibrosis [21]. Our data demonstrated that D-psicose improved insulin resistance through the protection of β-islets from injury through its strong anti-fibrotic effect and study on the mechanisms related to the causative factors underlying this anti-fibrotic effect of D-psicose is on-going.

However, in favor of anti-fibrotic effect Masson's staining (Fig. 4I–L) showed marked diminution of fibrosis with D-psicose treatment (Fig. 4O), whereas it was severe in control islets (Fig. 4J arrows). Additionally, we performed α-SMA immunostaining (Fig. 4E–H), where spindle-shaped cells expressing α-SMA protein found in the OLETF control rats (Fig. 4F) which closely correlated with the area of connective tissue proliferation. Whereas, this feature was very few and limited only to the vessel wall in D-psicose-treated rats, (Fig. 4G). These were our main findings in favor of D-psicose's antifibrotic effect although the underlying mechanism is yet to be elucidated. However, increased α-SMA expression within the islet might reveal the involvement of oxidative stress-induced pancreatic stellate cell activation in the development of islet fibrosis in OLETF control rats. We think this antifibrotic effect of D-psicose in the present study might be through the inhibition of oxidative stress since anti-oxidative effects of rare sugars have been investigated recently where D-psicose showed dose-dependent scavenging activity [22]. D-Psicose also has been shown to prevent DEHP-induced testicular injury by suppressing the generation of reactive oxygen species in rat testis [23].

The next issue is obesity, especially intra-abdominal fat, frequently involved in T2DM, and adipose tissue-mass enlargement has been considered to be closely associated with insulin resistance [24]. Adipocytes usually take up glucose and store energy in the form of triglycerides and secrete various bioactive molecules known as adipocytokines [25,26]. Of those, higher plasma levels of leptin, adiponectin, plasminogen activator inhibitor-1, and TNF-α accompanied obesity and participated in the development of insulin resistance are reported [27,28]. Therefore, adipocyte hypertrophy and adipocytokines are thought to be key pathological contributors to insulin resistance, increasing the risk of T2DM. Our results showed that D-psicose significantly reduced serum leptin level although adiponectin level was non-significantly higher (Fig. 1F). Also, we found that D-psicose had a significant effect on adipocyte morphology, indicated by the decreased size of adipocytes (Fig. 2C). These observed effects of D-psicose on the serum leptin level and white adipose tissue may add further support to the beneficial effect of D-psicose against T2DM by reducing the risk associated with obesity. In summary, this study presented strong evidence that rare sugar D-psicose provided protection of pancreas β-islets from the injury caused by sustained T2DM and thus controlled the progression of diabetes.

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