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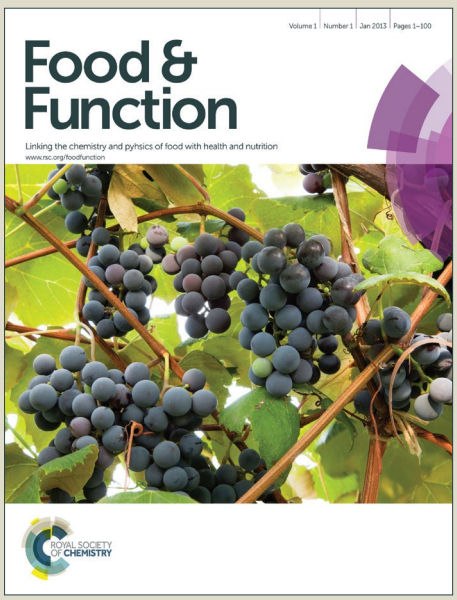

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Hypoglycemic and Hepatoprotective Effects of D-*Chiro*-Inositol-Enriched Tartary Buckwheat Extract in High Fructose-Fed Mice

Yuanyuan Hu[a], Yan Zhao[b,*], Daoyuan Ren[a], Jianjun Guo[a], Yiyang Luo[a], Xingbin Yang[a,*]

[a]Key Laboratory of Ministry of Education for Medicinal Resource and Natural Pharmaceutical Chemistry, College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi'an 710062, China

[b]School of Pharmacy, Fourth Military Medical University, Xi'an 710032, China

*Corresponding author: Tel.: +86-29-85310580; Fax: +86-29-85310517

E-mail address: xbyang@snnu.edu.cn (X.B. Yang), yanzhao@fmmu.edu.cn (Y. Zhao)

Abstract

This study was designed to investigate the protective effects of D-*Chiro*-Inositol (DCI) enriched tartary buckwheat extract (DTBE) against high fructose (HF) diet-induced hyperglycemia and liver injury in mice. HPLC analysis revealed that the content of DCI presented in purified DTBE was 34.06%. Mice fed 20% fructose in drinking water for 8 weeks significantly displayed hyperglycemia, hyperinsulinemia, dyslipidemia, hepatic steatosis and oxidative stress ($p<0.01$). Continuous administration of DTBE in HF-fed mice dose-dependently reduced the HF-induced elevation of body weight, serum glucose, insulin, total cholesterol (TC), total triglycerides (TG) and low density lipoprotein cholesterol (LDL-C) levels, as well as serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), C-reactive protein (CRP) and lactate dehydrogenase (LDH) activities, while the HF-induced decline of serum high density lipoprotein-cholesterol (HDL-C) levels could be markedly elevated in mice. Meanwhile, DTBE also dose-dependently increased the hepatic total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-P*x*) activities, and decreased hepatic malonaldehyde (MDA) levels, relative to HF-treated mice. Histopathology of H&E and Oil Red O staining confirmed the liver injury induced by a HF diet and the hepatoprotective effect of DTBE. These findings firstly demonstrate that intake of DTBE may be a feasible preventive or therapeutic strategy for HF diet-induced hyperglycemia, hepatic steatosis and oxidative injury.

Keywords: Tartary buckwheat, D-*Chiro*-Inositol, HPLC, Hyperglycemia, Liver injury

Introduction

Fructose as a sweetener in the food industry is primarily in the form of sucrose and high-fructose corn syrup (HFCS: 55-90% fructose content), and its consumption has been dramatically increased in recent years.[1] However, studies have shown that intake of high-fructose (HF) diet can cause insulin resistance (IR) associated with deleterious metabolic consequences including hyperglycaemia, dyslipidemia, excessive generation of reactive oxygen species (ROS), malfunctioning of the liver and non-enzymatic fructosylation of proteins.[2,3] IR is a prominent feature of metabolic syndrome and a potential contributor to type 2 diabetes and atherosclerosis.[4] In addition, animal studies have also shown that excessive fructose consumption results in unregulated hepatic fructose metabolism for the unlimited uptake and metabolism of hepatic fructose by inhibitory feedback mechanisms, which leads to hepatic steatosis and alters lipid metabolism.[5,6] Dietary fructose also causes inflammation and oxidative stress, which are implicated in the pathophysiology of insulin resistance.[7]

D-*chiro*-inositol (DCI) is a compound with an insulin-like bioactivity, which acts as a component of a putative mediator of insulin action.[8] It works to increase the action of insulin, and decrease blood pressure, plasma triglycerides, glucose concentration, and improve the function of ovary in the polycystic ovary syndrome (PCOS) women.[9,10] Some evidences suggest that DCI also has the function of anti-oxidation, anti-aging and anti-inflammation.[11] Additionally, chronic and acute treatment with DCI was shown to prevent and reverse endothelial damage by decreasing ROS and enhancing endothelial nitric oxide synthase (*e*NOS) and nitric oxide (NO) bioactivity in a dose-dependent manner.[12]

Tartary buckwheat (*Fagopyrum tataricum*) is an excellent source of several phytochemicals that have positively influences on managing diabetes mellitus and attenuating hyperglycemia.[13] Importantly, tartary buckwheat has also been demonstrated to be a vital natural source of DCI,[8] which contains more different galactosyl derivatives of DCI known as fagopyritols, and higher levels of free DCI compared to other buckwheats.[14] There are reports showing that

administration of the buckwheat as a natural source of DCI may significantly improve glucose tolerance in normal and streptozotocin (STZ) rats, and decrease hyperglycemia in STZ-fed rats.[14] It has also been reported that DCI-enriched tartary buckwheat bran extract may decrease the blood glucose level, modify favorably the lipid profile, and improve insulin immune reactivity in KK-A^y mice.[15] However, to our best knowledge, there are no available studies regarding the effects of natural DCI on hepatic steatosis and pathological development of liver injury induced by consumption of a high-frucose diet.

The present study was therefore designed to purify the DCI from Chinese tartary buckwheat by activated carbon column, and characterize it by high performance liquid chromatography (HPLC). Furthermore, we investigated the protective effects of DTBE on hyperglycemia, hepatic steatosis and oxidative injury in a well-established mouse model by feeding mice with a HF diet. This paper provided a clue for substantiating dietary and therapeutic use of tartary buckwheat in hyperglycemia and hepatic injury.

Materials and methods

Materials and Reagents

Tartary buckwheat flour from whole seeds was obtained from Ningqiang County Qiang State Food Co. Ltd. (Shaanxi, China). Activated carbon was the product of Tangshan Marine Chemical Co. (Hebei, China). DCI standard (99%) and trifluoroacetic acid (TFA, 99%) were purchased from Sigma-Aldrich (Shanghai, China). Food grade fructose was obtained from Senbo Biology Co., Ltd (Xi'an, China). Haematoxylin and eosin (H&E) and Oil red O were the products of Shanghai Lanji Technological Development Co. Ltd. (Shanghai, China). Detection kits for glucose, total cholesterol (TC), total triglyceride (TG), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Changchun Huili Biotechnology Co., Ltd. (Changchun, China). Assay kits of lactate dehydrogenase (LDH),

superoxide dismutase (SOD), glutathione peroxidase (GSH-P*x*) and malonaldehyde (MDA) were the products of Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enzyme linked immunosorbent (ELISA) kits of insulin and C-reactive protein (CRP) were also obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). Acetonitrile was purchased from Acros-Organic (Geel, Belgium). All other reagents and chemicals were of analytical grade.

Extraction of DCI

DCI fraction of tartary buckwheat was isolated according to the procedure described with some modifications.[16] In brief, the extraction was performed by mixing 50 g of the dried tartary buckwheat powder with 1.0 L of 50% ethanol aqueous solution at 50°C in one conical flask, and incubated in a water bath shaker under room temperature for 30 min, and this procedure was repeated three times. After vacuum filtration at 50°C, the supernatants were combined and concentrated under a reduced pressure using a rotary evaporator (RE-52AA, Shanghai Yarong Biochemical Equipment Co., Shanghai, China), and then lyophilized in the freeze-dry apparatus (Yuhua, china) to get the completely dried powder of the crude extracts.

Purification of DCI

Most DCI in buckwheat exists in the form of fagopyritols, and can be converted to DCI by acidic hydrolysis.[17] Therefore, 1.0 mL of dried extract was hydrolyzed by 2 mL of 3 N trifluoroacetic acid (TFA) in the water bath shaker at 70°C for 4 h.[16] After the hydrolysis, the sample was concentrated to one-third volume by the rotary evaporator, and then was passed through the activated carbons column (Φ 30 mm × 310 mm) to purify at the flow rate of 1.0 mL/min. After reaching adsorptive saturation, the column was firstly washed by deionized water, and then eluted by 4% ethanol with a flow rate of 1.0 mL/min. The eluate was pooled, followed by freeze-drying, and the dry powder was washed by deionized water and freeze-dried to obtain a white powder of DCI-enriched tartary buckwheat extract. The DCI-enriched tartary buckwheat

extract was named as DTBE in this study, and this part was further applied in the following experiments for the quantification of DCI and its protective effects on high-fructose diet-induced hyperglycemia and liver oxidative injury in mice.

HPLC analysis of DCI

The quantification of DCI in the preparation was carried out using a Acchrom XAmide column (4.6 mm i.d. × 150 mm, 5 μm) on a Shimadzu LC-2010A HPLC system equipped with an refractive index detector (RID-10A), an autosampler and a Shimadzu Class-VP 6.1 workstation software (SHIMADZU, Kyoto, Japan). The DCI standard and samples were all dissolved in 70% acetonitrile aqueous solution to yield stock solution at the concentrations of 0.18 mg/mL and 1.8 mg/mL, respectively. Before injection, all solutions were diluted and subsequently filtered through a 0.45 μm Millipore membrane. The mobile phase was composed of acetonitrile and distilled water (70:30, v/v), and the flow rate of the mobile phase was set at 1.0 mL/min. The sample injection volume was 20 μL at a 30°C column temperature, and RID condition was optimized to achieve the sensitivity of 512 nm.

Animals and experimental design

Healthy male Kunming mice (weight 18-22 g) were purchased from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). All animal experiments were conducted according to the ethical guidelines outlined in the Guide for Care and Use of Laboratory Animals. The animal facilities and experimental protocol were approved by the Committee on Care and Use of Laboratory Animals of the Fourth Military Medical University, China (SYXK-007-2007). Mice were acclimatized for at least 7 days prior to use and were housed under standard conditions with 12/12 h light-dark cycle at room temperature of 22 ± 2°C and humidity 60 ± 5%. They were fed standard rodent chow with water ad libitum and fasted over-night before the experiments. Group of experiments were carried out according to the previous method with some modifications.[15] Mice were randomly assigned to five groups of 10 animals in each group. Group I: mice received only tap water and were administered

intragastrically (ig.) with physiological saline during the experimental period (normal group) for 8 weeks. Group II: mice received 20% high-fructose water and were administered ig. with physiological saline (HF group). Group III: mice received high fructose water (20%, w/v) and were administered ig. with 40 $mg.kg^{-1}$.bw DTBE (equivalent to 13.62 $mg.kg^{-1}$.bw DCI). Group IV: mice received 20% high-fructose water and were administered ig. with 80 $mg.kg^{-1}$.bw DTBE (equivalent to 27.24 $mg.kg^{-1}$.bw DCI). Group V: mice received 20% high-fructose water and were administered ig. with 160 $mg.kg^{-1}$.bw DTBE (equivalent to 54.50 $mg.kg^{-1}$.bw DCI). The mice were allowed free access to tap water or 20% high-fructose water. DTBE was dissolved in redistilled water and administered ig. at 40, 80 and 160 mg/kg body weight once daily (0.4 mL) for 8 consecutive weeks. The mice from the normal and high-fructose groups were also given the same volume of vehicle, and 20% high-fructose water was renewed every other day. The body weight of all the groups was measured once a week. Food and water intake was monitored daily, and then the average food and water intake of each mouse in different groups was calculated. All the administrations were conducted between eight and nine o'clock in the morning once daily. Two hours after the last administration, all the animals were fasted overnight (12 hours) and anesthetized by the inhalation of isoflurane, and then sacrificed by cervical dislocation. Blood was withdrawn into a syringe from the abdominal aorta, and mouse liver was immediately removed and washed by ice-cold physiological saline.[18] The serum was separated by centrifuging the blood samples at 3500*g* for 15 min and then frozen at -20°C until use, while the livers were frozen at -80°C. On the basis of the records of the body weight and corresponding liver weight of every mouse, we calculated the hepatosomatic index (HI) according to the following formula: HI = liver weight/body weight × 100%. All the experiments were conducted according to the Guidelines of Experimental Animal Administration published by the State Committee of Science and Technology of People's Republic of China.

Oral glucose tolerance test (OGTT)

Mice were fasted for 12 h before they were orally administered with a freshly prepared

glucose solution at a dose of 2.0 g/kg body weight. Blood samples were collected from the caudal vein by means of a small incision at the end of the mouse tail, and blood glucose level was estimated using a commercial glucometer (Sannuo, China) at 0 (before glucose administration), 30, 60, 90 and 120 min after glucose administration. OGTT was performed on days 42 and 56. The area under the curve (AUC) of glucose during the glucose tolerance test was calculated by the trapezoidal method.[6]

Measurement of serum glucose and insulin

The serum glucose was measured by the glucose oxidase method.[19] The serum insulin concentrations were determined by the competitive inhibition method of ELISA assay according to the kit manufacturer's instructions. The homeostasis model assessment of basal insulin resistance (HOMA-IR) and β-cell function (HOMA-β) were calculated according to the following formulas: HOMA-IR = [fasting serum insulin (mU/L) × fasting serum glucose (mmol/L)]/22.5, HOMA-β = [20 × Fasting serum insulin (mU/L)]/[Fasting serum glucose (mmol/L) – 3.5].[20] Higher HOMA-IR and lower HOMA-β levels accounted for lower insulin sensitivity and β cell function, respectively.[7]

Measurements of serum lipid profile, ALT, AST, LDH and CRP levels

The measurements for fasting serum TC, TG, LDL-C and HDL-C concentrations were conducted by enzymatic colorimetric methods using commercial kits, and the results were all expressed in mmol/L. The serum enzymic activities of ALT and AST were measured by commercially available diagnostic kits. The enzymatic activities were expressed as units per litre (U/L). The measurement of LDH activity was based on its ability to catalyze the reduction of pyruvate, in the presence of NADH, to form lactate and NAD^+ using a commercial kit. The serum CRP was estimated by the competitive inhibition method of ELISA assay according to the kit manufacturer's instruction. The enzymatic activities of LDH and CRP were expressed as U/L and ng/mL, respectively.

Measurement of hepatic MDA, T-SOD and GSH-P*x* levels

The liver tissue was homogenized (10%, w/v) in ice-cold 50 mM phosphate buffer (pH 7.4) by an automatic homogenizer (F6/10-10G, FLUKO Equipment Shanghai Co. Ltd., Shanghai, China). During the preparation, 0.5 g of each hepatic tissue was homogenized in 9-fold frozen normal saline in volume, and centrifuged at 5000*g* and 4°C for 15 min. The supernatant was obtained and used for the measurements of MDA, T-SOD and GSH-P*x*. The protein concentration in homogenates was assayed by the method of Coomassie brilliant blue.[18] The analysis for hepatic MDA level was performed with commercially available diagnostic kits, and the result was expressed as nmol/mg protein. GSH-P*x* and T-SOD activities were assessed using common commercial kits, and the results were expressed as U/mg protein.

Histopathological observation of livers

Histology of the liver was examined through H&E and Oil red O staining. For H&E staining, a portion of the liver from the left lobe was fixed in a 4% paraformaldehyde solution. Fixed tissues were embedded in paraffin, cut into slices (5-6 μm thick), and then stained with H&E dye, and observed under a light microscope (DM-LB2, Leica, Germany) for detection of hepatic damage. For Oil Red O staining, the liver sample was processed using cryostat (CM1950, Leika, Germany) and then fixed and stained. The stained area was detected under an Olympus light microscope at 400 ×. Finally, the images were examined and evaluated for pathological change analysis.

Statistical Analysis

All of the experiments were performed in triplicate and the data were expressed as means of ± SD (standard deviation). All data were analysed by one way analysis of variance (ANOVA) followed by Duncan's multiple-range tests (SPSS, version 13.0). The p-value < 0.05 was considered statistically significant.

Results

Chemical properties of DCI

DCI was extracted from tartary buckwheat powder with 50% ethanol extraction, and the content of DCI in the crude extract reached only 0.026% (w/w). The DCI-enriched tartary buckwheat extract (DTBE) was further obtained from the crude extract by acidic hydrolysis and a separation on the activated carbons column. With this method, the extraction yield of DTBE was approximately 0.83% (w/w) of the dried tartary buckwheat powder. Furthermore, a routine HPLC chromatographic procedure was performed to determine DCI in the preparations. Fig.1 A displayed the typical HPLC-RID chromatogram of single DCI profile in purified DTBE, and the HPLC-RID chromatogram of DCI standard was shown in Fig. 1B. The identification of DCI was performed according to the retention time (t_R) obtained from authentic standard under identical HPLC condition. As depicted in Fig. 1B, a well-defined peak ascribed to DCI was detected at a suitable t_R. In this study, the correlation between DCI concentration and area response was best described by the following equation: Y = 1.1E+7X + 2E+5 (R^2 = 0.99, n = 5; Y = peak area of analyte; X = concentration of analyte). As shown in Fig. 1 A, HPLC analysis clearly indicated that a well separation between DCI and other components was obtained in the DTBE with an outstanding DCI peak. A further enrichment through column chromatography of activated carbon could produce DTBE with DCI of up to 34.06%, suggesting that the purity of DCI may be well improved by purification with activated carbon.

Effects of DCI on liver and body weights, and liver index

After giving 20% fructose water for 8 weeks, body weight, liver weight and HI of the tested mice were investigated among groups. As shown in Table 1, the mice fed a high-fructose diet showed a significant increase in body weight ($p < 0.05$), liver weight ($p < 0.01$) and HI ($p < 0.01$), when compared to the normal diet group after 8 weeks. Interestingly, the increased body weight could be well decreased by the oral administration of middle- and high- doses of DTBE ($p < 0.05$, $p < 0.01$). Additionally, treatment with DTBE at 80 and 160 mg/kg·bw also significantly attenuated the increases in liver weight and HI of HF-treated mice ($p < 0.01$). However, co-treatment with DTBE at a low dose led to slight decreases in body weight, liver

weight and HI, and there was no statistical significance ($p > 0.05$). It was also found that the mean food consumption (an average of 7 g/mice/day) and water ingestion were not significantly different among all the tested groups (data not shown). The present result suggests that DTBE can effectively inhibit the HF-induced body weight and liver weight gain.

Oral glucose tolerance test (OGTT)

The response to an oral glucose tolerance test on days 42 and 56 is shown in Fig. 2 A-D. After animals received a glucose load orally, incremental plasma glucose concentrations peaked at 30 min. It was worth noting that after 120 min of glucose administration, all the DTBE-supplemented mice showed extremely significant lower glucose level compared to the HF group ($p < 0.01$). Serum glucose concentrations were significantly greater in HF-treated mice, and remained higher at all the tested points throughout the OGTT study period compared to the normal mice (Fig. 2A and C). In addition, fructose feeding impaired glucose tolerance, where the area under the curve (AUC) in the HF-treated mice at the corresponding time on day 42 and 56 was elevated approximately 49.49% and 55.66%, respectively, relative to the normal group ($p < 0.01$, Fig. 2B and D). As expected, the HF-induced increases were effectively attenuated on day 42 and 56 by the co-treatment with DTBE at the tested dosages of 40, 80, and 160 mg/kg·bw in a dose-dependent manner, respectively ($p < 0.01$), indicating that DTBE improved the glucose intolerance of the mice with chronic consumption of 20% HF water.

Effects of DCI on serum glucose, insulin concentrations, HOMA-IR and HOMA-β

The levels of glucose, insulin, HOMA-IR and HOMA-β are shown in Table 1. The glucose and insulin levels in HF-treated mice were sharply increased to 10.03 ± 0.90 mmol/L and 28.20 ± 1.95 mU/L from 8.05 ± 1.17 mmol/L and 22.71 ± 2.32 mU/L of the untreated normal mice, respectively ($p < 0.01$). However, the protective administration of medium- and high-doses of DTBE significantly abolished the HF-elevated levels of serum glucose and insulin ($p < 0.01$). Furthermore, high fructose induced substantial reduction in insulin sensitivity and β cell function as indicated by the significantly high HOMA-IR ($p < 0.01$) and low HOMA-β ($p < 0.05$)

indices in mice, relative to the normal control mice. Interestingly, the HOMA-IR score of the mice treated with DTBE at 40, 80 and 160 mg/kg·bw showed a significant decrease by 15.48% ($p < 0.05$), 32.46% ($p < 0.01$) and 37.14% ($p < 0.01$), relative to HF-fed mice, respectively. Meanwhile, the decreased HOMA-β index in HF-treated mice was also effectively attenuated by the co-treatment with high dosages of DTBE ($p < 0.05$), while the administration of low- and medium-doses of DTBE failed to show significant decrease ($p > 0.05$).

Effects of DCI on serum lipid profiles

As shown in Table 2, high fructose consumption caused dyslipidemia, as evidenced by a significant increase in TC, TG and LDL-C from 4.03 ± 0.62 mmol/L, 2.48 ± 0.32 mmol/L, and 2.13 ± 0.16 mmol/L in untreated normal group to 6.55 ± 0.71 mmol/L, 2.99 ± 0.39 mmol/L, and 2.73 ± 0.28 mmol/L, respectively ($p < 0.01$), and a remarkable decrease in serum HDL-C from 1.77 ± 0.20 mmol/L to 1.39 ± 0.21 mmol/L was observed ($p < 0.01$). Administration of medium- and high- doses of DTBE effectively protected against the increases in serum TC, TG and LDL-C, and against the reduction of the serum HDL-C level in a dose-dependent manner in HF-induced hyperlipidemic mice. While the co-treatment with low dose of DTBE showed slight changes in TC, TG, LDL-C and HDL-C, with no statistical significance ($p > 0.05$).

Effects of DCI on serum ALT, AST, LDH activities and CRP levels

As represented in Table 2, the enzymatic activities of serum ALT, AST and LDH in HF-induced mice were remarkably increased to 94.89 ± 7.88 U/L, 144.61 ± 10.35 U/L and 885.20 ± 137.93 U/L from 77.31 ± 8.45 U/L, 101.16 ± 7.37 U/L and 191.01 ± 38.23 U/L of the normal mice, respectively ($p < 0.01$). However, co-treatment of DTBE at 80 and 160 mg/kg·bw significantly reduced the HF-induced elevation of serum ALT activities by 13.62% ($p < 0.05$) and 29.30% ($p < 0.01$), respectively. Meanwhile, a HF-induced increase in AST and LDH activities was also effectively attenuated by the co-treatment with DTBE at all the tested dosages ($p < 0.01$), suggesting that DTBE exhibited strong protective effects against HF-induced hepatotoxicity. Furthermore, fructose-enriched diet caused inflammation as

indicated by a significant increase in serum CRP level of HF-diet mice from 106.42 ± 10.91 ng/mL to 132.09 ± 19.57 ng/mL ($p < 0.01$, vs normal group, Table 2). However, the HF-induced elevation of serum CRP was effectively attenuated by the supplementation of all doses of DTBE, relative to HF-fed mice, respectively ($p < 0.01$). As shown in Table 2, treatment with DTBE at 40, 80 and 160 mg/kg·bw significantly lowered the serum CRP levels compared with HF group by 19.95%, 23.34% and 34.83%, respectively ($p < 0.01$).

Effects of DTBE on hepatic MDA, T-SOD and GSH-P*x* levels

As shown in Fig. 3A, the hepatic MDA was significantly increased from 2.51 ± 0.38 nmol/mgprot of the normal mice to 4.99 ± 0.44 nmol/mgprot of the HF-fed mice ($p < 0.01$). However, this HF-induced increase was effectively attenuated by the co-treatment with DTBE at all the tested dosages ($p < 0.01$). Furthermore, continuous feeding of HF in mice caused characteristic hepatotoxicity in antioxidant parameters of liver tissue, as reflected by a 31.4% decrease of hepatic T-SOD activity ($p < 0.01$) and a 29.5% decrease of hepatic GSH-P*x* activity ($p < 0.01$) in the mice fed 20% HF water (Fig. 3B and C). However, HF-induced decrease in T-SOD activity was prevented by the oral supplementation with DTBE at 80 ($p < 0.05$) and 160 mg/kg·bw ($p < 0.01$), and this protective effect could be performed in a dose-dependent manner. Meanwhile, the hepatic GSH-P*x* activity was also effectively increased by the co-treatment with DTBE at all the tested dosages of 40 ($p < 0.05$), 80 ($p < 0.01$), and 160 mg/kg·bw ($p < 0.01$), respectively.

Histopathological examination of mouse livers

Histopathological observation of H&E and Oil Red O staining of the livers was performed to further support the evidence for the biochemical analysis (Fig. 4). For H&E staining, the liver slices of normal mice showed typical hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus, and visible central veins (Fig. 4A). However, the liver sections of HF-fed mice showed severe cellular degeneration, hepatocyte necrosis, cytoplasmic vacuolation and the loss of cellular boundaries (Fig. 4B). As expected, DTBE dose-dependently reduced the hepatic

317 injuries caused by HF feeding, showing near normal appearance with well-preserved cytoplasm,
318 prominent nuclei, and legible nucleoli (Fig. 4C-E). For Oil Red O staining, in contrast to the
319 normal appearance of hepatic tissues in control group (Fig. 4F), the livers of HF-fed mice
320 showed widespread deposition of lipid droplets inside the parenchyma cells (Fig. 4G).
321 Nevertheless, these vacuolization and fatty changes of hepatocytes were alleviated in the
322 DTBE-treated mice, showing slight scattered droplets of fat, and this protective effect was
323 dose-dependent (Fig. 4H-J). The results together with biochemical tests suggested that DTBE
324 could protect liver tissues from HF-induced liver damage in mice.
325

326 ## Discussion

327 Dietary fructose is a caloric monosaccharide which can induce metabolic disorders including
328 insulin resistance, hyperinsulinemia, hypertension, obesity, dyslipidemia, type 2 diabetes
329 mellitus and atherosclerosis.[21,22] The development of insulin resistance and other related
330 diseases in high fructose-fed mice is well documented in the literatures.[4,6] Herein, our
331 experimental results were consistent with previous studies which found that HF-fed animals
332 presented hyperglycemia, hypertriglyceridemia, glucose intolerance, hepatic steatosis and
333 impaired antioxidant potential and consequently, a reduction of insulin sensitivity.[4,23] Previous
334 study has demonstrated that DCI is a useful compound with an insulin-like bioactivity and is
335 able to reduce meal-induced hyperglycemia.[15] In this regard, the DCI derived from tartary
336 buckwheat is expected to play a promising role in protection against HF-induced insulin
337 resistance and its related diseases. Herein, DCI was successfully isolated from tartary buckwheat
338 by ethanol extraction with acidic hydrolysis and enrichment through activated carbon column,
339 and the obtained DTBE was shown to contain 34.06% of DCI by HPLC analysis. Furthermore,
340 DTBE was firstly demonstrated to exhibit protective effects against HF-induced metabolic
341 syndromes of hyperglycemia and liver dysfunction by mitigating hepatic oxidative damage and
342 inhibiting liver steatosis in mice.

In the present study, the mice fed 20% high fructose water for 8 consecutive weeks exhibited a significantly impaired ability of insulin to stimulate glucose disposal, associated to a compensatory hyperinsulinemia, as evidenced by elevations of fasting glucose and insulin levels and an increase of HOMA-IR index in HF group. This treatment also resulted in a decline of basal β-cell function, reflected by the decrease of HOMA-β index, which might expectedly occur as a result of the sustained load of β-cells to compensate for insulin resistance.[24] However, administration of DTBE significantly prevented the HF-induced increases in circulating glucose, insulin, AUC and HOMA-IR, and enhanced the values of HOMA-β index to the levels that approached those of normal control mice. The mechanism of the antihyperglycemic effect of DTBE might contribute to the protective effect on pancreatic islet cells, and the increase of insulin secretion from the remaining pancreatic β-cells.[25]

High fructose-induced insulin resistant status is commonly characterised by a profound metabolic dyslipidemia, accompanied by increased LDL-C, high serum TC and TG, and low HDL-C levels.[26] Results of the present study showed that a HF diet induced an elevation of serum TC, TG, LDL-C and a reduction of serum HDL-C levels, which was consistent with the previous studies.[7,23] In addition, the weight of the body and the liver, and hepatosomatic index were all higher in the HF-diet mice than that in the normal mice. It is well known that excess fructose in the diet can cause glucose malabsorption and elevation in synthesis of TG when compared to other carbohydrates for its lipogenic properties.[27] However, co-treatment of DTBE appeared to have remarkable protective effects against dyslipidemia induced by HF and prevented the increases in body and liver weights in mice, which might be through normalizing the neuroendocrine pattern which was consistent with diet-induced obesity,[28] or probably resulted from a decrease in lipid synthesis with regulation of the glycolytic pathway.[29]

Furthermore, the results of histopathological observation of representative Oil Red O staining of the livers confirmed the lipid abnormalities in HF-fed mice, characterized by widespread deposition of lipid droplets inside the hepatic parenchymal cells (Fig. 4G), which were

consistent with the result of the biochemical analysis. However, the liver of DTBE-treated mice showed scattered droplets of fat in comparison with HF-fed mice. These results surprisingly suggest that DTBE has the effect of promoting liver fat metabolism and may play a protective role against hepatic steatosis induced by a high-fructose diet.

ALT and AST activities are reliable markers of liver function, where the increased ALT activity is an indicator of cell membrane damage and the elevated AST activity is another indicator of mitochondrial damage.[30] Serum LDH is a well-documented biochemical marker of hepatic dysfunction by the metabolism problem and cell loss in the liver.[31] The current results presented in this study showed that application of 20% high frucose water markedly raised serum ALT, AST and LDH activities in mice. Interestingly, DTBE-treated mice effectively corrected the status of liver dysfunction by reducing the activities of ALT, AST and LDH in a dose-dependent manner, which represented the preventive action of DTBE against liver damage in HF-fed mice. Furthermore, CRP is a plasma protein synthesized by the liver, and is a marker of systemic inflammatory response and tissue damage.[32] Recent studies have shown that elevation of CRP concentrations is a risk factor for insulin resistance and metabolic syndrome (MetS).[7] In our study, HF-fed mice showed the marked inflammation as evident from the increased serum CRP levels. However, the treatment with DTBE exerted the remarkably anti-inflammatory effects, reflected by decreased serum CRP levels.

Fructose-induced hyperglycemia is one of the important factors to increase ROS, and subsequent lipid peroxidation causes the depletion of the antioxidant defense status in various tissues.[23] In addition, it has been postulated that fructose can accelerate free radical production similar to glucose, and ROS can reduce the activity of antioxidant enzymes.[3] SOD and GSH-P*x* are the major natural antioxidant enzymes which play an important role in the elimination of ROS derived from the redox process in liver tissues.[33] MDA is the final stage of lipid peroxidation of the polyunsaturated fatty acid of biological membrane, which can result in failure of the antioxidant defense mechanisms to prevent the formation of excessive ROS.[34] In

our study, high fructose feeding caused significant oxidative stress, indicated by significantly low SOD and GSH-P*x* activities and high MDA concentrations in the HF-fed mice, relative to the untreated normal mice. These findings are consisted with other investigations where there is a significant increase in lipid peroxidation or a significant decrease of hepatic antioxidant enzyme activities in fructose-induced diabetic rats.[3,6] However, oral administration of DTBE dose-dependently prevented the liver oxidative stress damage via normalizing SOD, GSH-P*x*, and MDA levels, and these protective effects may be due to the ability of DCI oral supplementation to reduce cellular ROS generation associated with chronic fructose feeding.[11] The present study also suggests that DTBE possesses antioxidant activity against hyperglycemia-mediated oxidative stress in liver. In parallel, several studies have also reported that D-pinitol, an inositol substance, can exhibit protective effect against oxidative damage in animal models of diabetes.[35,36] Moreover, histopathological examination of the HF-fed mice further showed distinct necrosis, ballooning degeneration, and inflammatory cell infiltration of the liver. However, these histological alterations were observably attenuated by DTBE, especially at dosage of 160 mg/kg·bw, showing nearly normal cellular architecture with distinct hepatic cells, which might be due to its prevention of the toxic chemical reactions from the formation of highly ROS induced by high fructose ingestion (Fig. 3E). These long-term hepatoprotective effects of DTBE in the present study might also be a consequence of additional antioxidant and anti-inflammatory effects of DCI,[11] and this is the first investigation with unequivocal evidence that DTBE can inhibit the HF diet-induced hepatic oxidative injury in mice. Moreover, it is of great significance to identify the beneficial effects of DCI as a natural compound present in tartary buckwheat against HF-induced metabolic disorders, which may be worthwhile as recent evidence to create a large opportunity for developing new functional products.

In conclusion, the present study clearly indicated the beneficial effect of DTBE against HF-induced hyperglycemia, dyslipidemia, hepatic steatosis and oxidative stress in mice. DTBE

421 was firstly demonstrated to possess the hepatoprotective effects against HF-caused
422 hyperglycemia and liver oxidative injury in mice, which might be due to its capability to prevent
423 high fructose diet induced oxidative stress and anti-inflammatory effects. All these findings
424 provide additional evidences in support of the use of DTBE as a promising traditional functional
425 food for the prevention and/or management of insulin resistance, liver damage and other related
426 metabolic disorders.
427

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Figure Captions

Fig. 1. Separation and detection of D-*chiro*-inositol by HPLC-RID. (**A**) Chromatogram of DCI-enriched tartary buckwheat extract (DTBE). (**B**) Chromatogram of DCI standard. HPLC analysis was carried out as described in the experimental section.

Fig. 2. Effects of DTBE on glucose tolerance on days 42 (**A**) and 56 (**C**), and total area under the curves (AUC) of plasma glucose on days 42 (**B**) and 56 (**D**) of 20% high fructose water-fed mice for consecutive 8 weeks, respectively. Data are expressed as means ± SD for 10 mice in each group. $^{\#\#}$ $p<0.01$, vs the normal group. *$p<0.05$, **$p<0.01$, compared to the HF group.

Fig. 3. Effects of DTBE on hepatic MDA (**A**), T-SOD (**B**) and GSH-P*x* (**C**) in the mice fed high frucose for 8 consecutive weeks. Values are expressed as means ± SD of 10 mice in each group. $^{\#\#}p<0.01$, vs the normal mice. *$p<0.05$, **$p<0.01$, compared to HF-treated mice.

Fig. 4. Effects of DTBE on the liver histological changes in HF fed mice for consecutive 8 weeks (original magnification of 400 ×). (**A**) The H&E staining of the normal group, (**B**) HF group, (**C**) 40 mg/kg·bw DTBE (low-dose) + HF diet, (**D**) 80 mg/kg·bw DTBE (medium-dose) + HF diet, (**E**) 160 mg/kg·bw DTBE (high-dose) + HF diet, (**F**) The oil-red-O staining of the normal group, (**G**) HF group, (**H**) 40 mg/kg·bw DTBE (low-dose) + HF diet, (**I**) 80 mg/kg·bw DTBE (medium-dose) + HF diet, (**J**) 160 mg/kg·bw DTBE (high-dose) + HF diet.

Table 1

Effects of DTBE on body weight, liver weight, and hepatosomatic index (HI), as well as glucose, insulin, HOMA-IR, and HOMA-β of the mice fed 20% high fructose (HF) water for consecutive 8 weeks.

Paramers	Normal	HF	HF + DTBE (40)	HF + DTBE (80)	HF + DTBE (160)
Initial body wt (g)	28.26 ±1.40	28.94 ± 1.45	28.16 ± 1.22	28.30 ± 2.02	27.96 ± 1.93
Final body wt (g)	46.15 ± 2.58	49.89 ± 2.32$^{\#}$	48.12 ± 1.78	46.75 ± 1.98*	45.75 ± 3.28*
Liver wt (g)	2.07 ± 0.17	2.53 ± 0.11$^{\#\#}$	2.46 ± 0.10	2.15 ± 0.17**	2.06 ± 0.11**
HI (%)	4.50 ± 0.33	5.19 ± 0.36$^{\#\#}$	5.13 ± 0.28	4.66 ± 0.46**	4.49 ± 0.36**
Glucose (mmol/L)	8.05 ± 1.17	10.03 ± 0.90$^{\#\#}$	9.35 ± 0.65	8.34 ± 0.92**	8.18 ± 0.89**
Insulin (mU/L)	22.71 ± 2.32	28.20 ± 1.95$^{\#\#}$	25.54 ± 2.64	23.06 ± 2.26**	21.96 ± 2.54**
HOMA-IR	8.16 ± 1.64	12.60 ± 1.73$^{\#\#}$	10.65 ± 1.61*	8.51 ± 0.92**	7.92 ± 0.61**
HOMA-β	110.21 ± 22.34	75.89 ± 12.12$^{\#}$	87.77 ± 8.29	99.01 ± 24.25	113.86 ± 34.58*

Values are expressed as means ± SD of 10 mice in each group.

$^{*}p < 0.05$, compared with the HF-fed mice.

$^{**}p < 0.01$, compared with the HF-fed mice.

$^{\#}p < 0.05$, as compared with the untreated normal mice.

$^{\#\#}p < 0.01$, as compared with the normal mice.

Table 2

Effects of DTBE on serum TC, TG, LDL-C, HDL-C, ALT, AST, LDH and CRP of high fructose-fed mice at the end of week 8.

Paramers	Normal	HF	HF + DTBE (40)	HF + DTBE (80)	HF + DTBE (160)
TC (mmol/L)	4.03 ± 0.62	6.55 ± 0.71$^{\#\#}$	5.86 ± 0.79	5.58 ± 0.72*	5.31 ± 0.92*
TG (mmol/L)	2.48 ± 0.32	2.99 ± 0.39$^{\#\#}$	2.65 ± 0.34	2.43 ± 0.28**	2.20 ± 0.43**
LDL-C (mmol/L)	2.13 ± 0.16	2.73 ± 0.28$^{\#\#}$	2.51 ± 0.25	2.33 ± 0.12**	2.25 ± 0.26**
HDL-C (mmol/L)	1.77 ± 0.20	1.39 ± 0.21$^{\#\#}$	1.51 ± 0.24	1.65 ± 0.12*	1.78 ± 0.17**
ALT (U/L)	77.31 ± 8.45	94.89 ± 7.88$^{\#\#}$	87.51 ± 10.81	81.97 ± 6.85*	67.28 ± 8.04**
AST (U/L)	101.16 ± 7.37	144.61 ± 10.35$^{\#\#}$	113.07 ± 9.43**	106.15 ± 8.85**	93.56 ± 9.38**
LDH (U/L)	191.01 ± 38.23	885.20 ± 137.93$^{\#\#}$	497.67 ± 112.70**	413.19 ± 67.97**	250.33 ± 65.62**
CRP (ng/mL)	106.42 ± 10.91	132.09 ± 19.57$^{\#\#}$	105.74 ± 13.07**	101.26 ± 10.96**	86.08 ± 16.35**

Values are expressed as means ± SD of 10 mice in each group.

$^{*}p < 0.05$, compared with the HF-fed mice.

$^{**}p < 0.01$, compared with the HF-fed mice.

$^{\#\#}p < 0.01$, as compared with the normal mice.

Fig. 1.

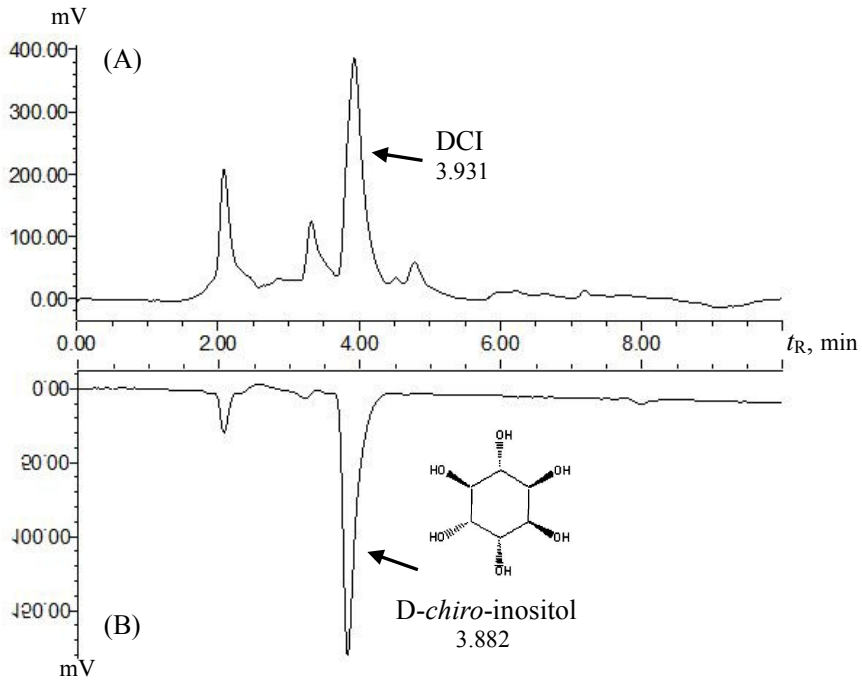

Fig. 2.

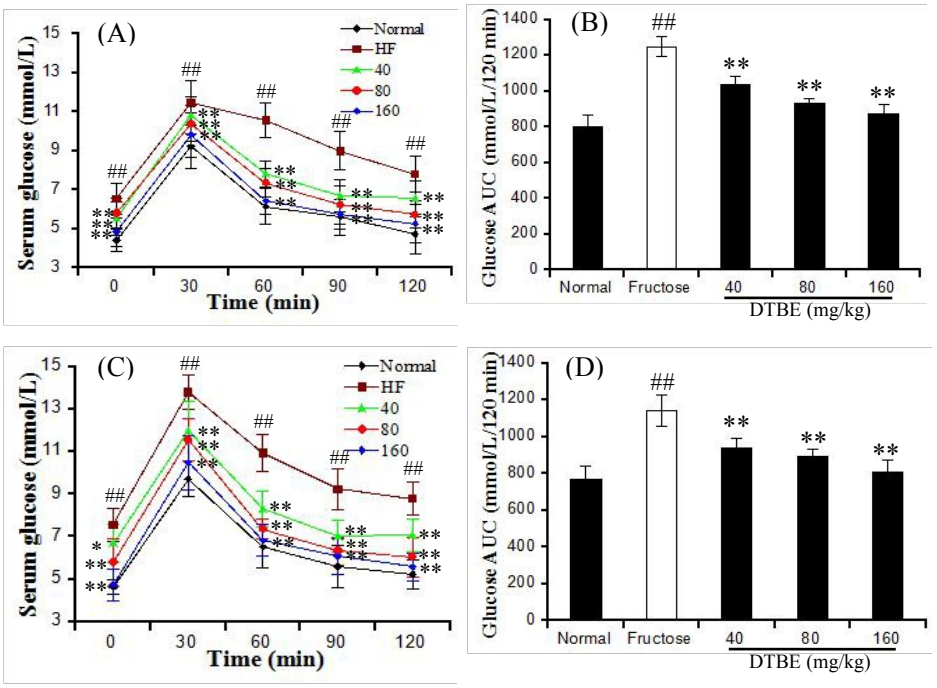

Fig. 3.

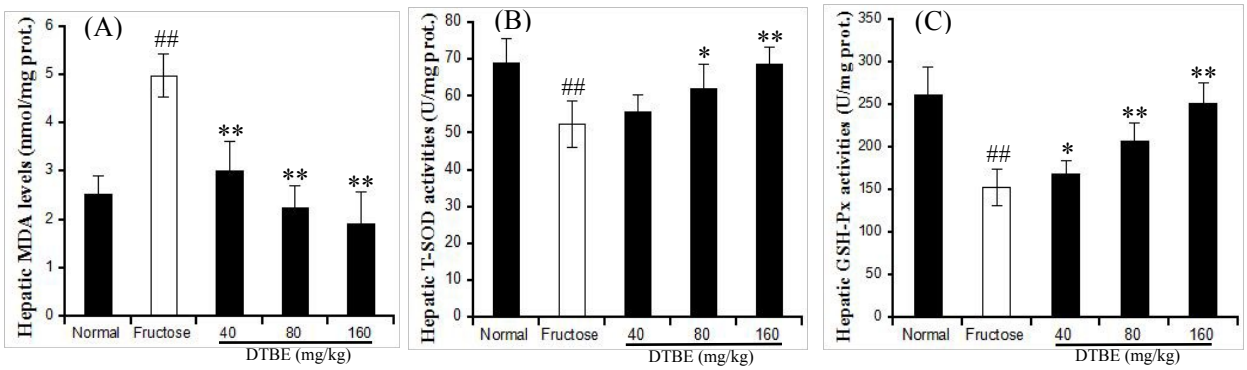

Fig. 4.

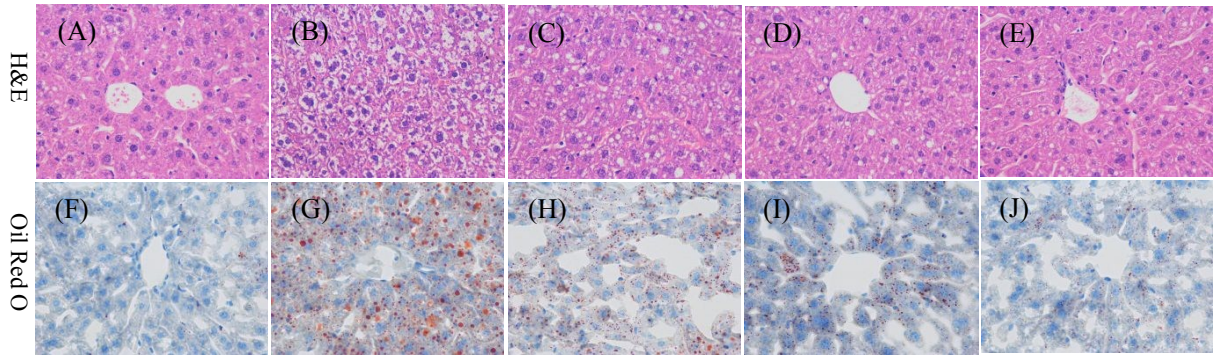

Table of Contents entry

D-Chiro-Inositol-Enriched Tartary Buckwheat Extract (DTBE) prevent the high fructose-induced hyperglycemia and hepatic injury in mice.