

## Short Communication

# Antioxidative and hepatoprotective effects of fructo-oligosaccharide in D-galactose-treated Balb/cJ mice

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### Abstract

Chronic subcutaneous (s.c.) administration of D-galactose (DG) to BL/6J mice has been shown to induce oxidative stress and is considered a model to mimic accelerated ageing. Fructo-oligosaccharide (FO) is a well-defined prebiotic and its fermentation by lactic acid bacteria has been shown to exert antioxidative capacity. The present study was aimed to determine whether FO attenuated DG-induced oxidative stress and hepatopathy in Balb/cJ mice. Mice (12 weeks of age, *n* 40) were divided into control (s.c. saline), DG (s.c. 1.2 g/kg body weight), DG + FO (5%, w/w) and DG + vitamin E (0.2%, w/w) groups and were killed after 52 d of treatment. Results indicated that DG significantly decreased the hepatic superoxide dismutase and glutathione peroxidase activities. These alterations were ameliorated both by FO and vitamin E. DG increased the hepatic TAG content approximately by 7.2% compared with the vehicle control, which was in agreement with the histological alteration. FO, similar to vitamin E, almost normalised the hepatic TAG content and ameliorated the histological characteristics of fatty liver. Similarly, the increased plasma alanine aminotransferase activity induced by DG was normalised by FO and vitamin E, respectively. Faecal bifidobacteria counts were greater in the DG + FO and DG + vitamin E groups compared with the DG group, respectively. In conclusion, the present study indicated that FO diminished the altered hepatic antioxidative enzyme activities and morphology caused by chronic DG administration in Balb/cJ mice, partially associated with its prebiotic role in the colon.

**Key words:** D-Galactose: Fructo-oligosaccharide: Antioxidative capacity: Liver: Ageing

One of the well-recognised ageing theories indicates that generation of oxidative stress leads to cell and tissue damage and ultimately results in ageing and cell death<sup>(1)</sup>. Chronic administration of D-galactose (DG) to BL/6J mice has been shown to induce changes which resemble accelerated ageing, such as formation of advanced glycation end products<sup>(2,3)</sup>, neurological impairment<sup>(4,5)</sup>, decreased serum antioxidative enzyme activities<sup>(4,5)</sup> and inflammation in the liver<sup>(3,6)</sup>. Several antioxidants<sup>(3,7,8)</sup> and Chinese herbs<sup>(9)</sup> have been shown to attenuate the ageing damage in C57BL/6J mice treated with DG.

Fructo-oligosaccharide (FO), a well-defined prebiotic<sup>(10)</sup>, has been incorporated into drinks and desserts to improve bowel function in elderly persons<sup>(11)</sup>. Recent clinical studies have initially shown that the consumption of a pre-

biotic mix<sup>(12)</sup> and a FO supplement<sup>(13)</sup> beneficially reduces the blood indices of peroxidation status. However, the effect of FO in DG-induced hepatic oxidative damage has never been demonstrated.

The main goal of the present study was to assess the anti-ageing and hepatoprotective effects of FO in DG-administered Balb/cJ mice, by the determination of antioxidative enzyme activities and the morphology of the liver and the biochemical indices of liver function.

### Materials and methods

#### Animals and diets

Male Balb/cJ mice (10 weeks old) were obtained from the BioLASCO Taiwan Company Limited (Taipei, Taiwan,

**Abbreviations:** DG, D-galactose; FO, fructo-oligosaccharide.

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ROC). After acclimatisation for 2 weeks, mice ( $n$  40) were randomly divided into four groups ( $n$  10): control (subcutaneous saline, basal diet), DG (subcutaneous 1.2 g D-galactose/kg body weight, basal diet), DG + FO (50 g active ingredients/kg basal diet) and DG + vitamin E (2 g  $\alpha$ -tocopherol/kg basal diet, as an antioxidant positive control) and were killed after 52 d of treatment. The basal diet consisted of a ground rodent chow (Lab 5001; Purina Mills, St Louis, MO, USA) and sucrose that match the digestible sugar present in the FO syrup (Institute of Microbial Resources, Taichung, Taiwan, ROC)<sup>(11)</sup>. The mixed powder diet was then re-formed into a small dough with deionised water in order to balance the liquid content among diets and to reduce spillage. The doses of supplements were used based on previous studies, indicating that supplementing 5% (w/w) FO into a fibre-free diet beneficially modulated colonic microflora and reduced faecal toxicity<sup>(14)</sup>, while 0.2% (w/w)  $\alpha$ -tocopherol altered pro-oxidation status in rats<sup>(15)</sup>. All animals were allowed to have free access to water and food during the study. Animal care followed the guidelines of the National Research Council<sup>(16)</sup> and was approved by the Institutional Animal Care and Use Committee in the Chung Shan Medical University. The mice were placed in metabolism cages during days 44–49, while fresh faeces were collected and frozen within 30 min of excretion. Faecal samples were lyophilised and kept at  $-20^{\circ}\text{C}$  for further analyses of microflora. The mice were anaesthetised with  $\text{CO}_2$  on day 52 after a 20 h fasting. Blood samples collected from the right atrium into heparinised tubes were centrifuged at 3000 g for 10 min and plasma samples were stored at  $-20^{\circ}\text{C}$  for the analysis of alanine aminotransferase activity. The mice were transcardially perfused with ice-cold normal saline ( $n$  6) or neutral formalin ( $n$  4) for 5 min or until the liver turned grey. Heparinised blood samples and livers, dissected from saline-perfused mice were frozen immediately for further analyses of enzyme activities, while those dissected from neutral formalin-perfused mice were fixed further with Bouin's solution overnight and then processed for histological routine.

#### *Hepatic antioxidative enzymes*

A portion of each liver was homogenised in 10 vol (v/w) of phosphate buffer (0.1 M, pH 7.4, containing 1 mM-EDTA). The homogenate was centrifuged at 10 000 g for 15 min at  $4^{\circ}\text{C}$  and the supernatant was immediately analysed. Superoxide dismutase activity was measured based on competition for superoxide radicals between superoxide dismutase and tetrazolium salt<sup>(17)</sup>. Glutathione peroxidase activity was measured indirectly by a coupled reaction with glutathione reductase that converted the oxidised glutathione to its reduced form with a concomitant oxidation of NADPH to  $\text{NADP}^+$ <sup>(18)</sup>. The catalase activity was measured colorimetrically based on the transformation of methanol to formaldehyde using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole<sup>(19)</sup>. Protein contents were analysed

based on the Bradford method<sup>(20)</sup>, using a protein assay reagent (Life Science Research, Hercules, CA, USA). Enzyme activity was expressed as IU/mg protein.

#### *Hepatic TAG content*

A portion of each liver was homogenised and extracted with 20 vol (v/w) of a chloroform–methanol mixture (2:1, v/v), according to Folch *et al.*<sup>(21)</sup>. Aliquots of lipid extracts were dried under vacuum and then dissolved in 95% ethanol. Hepatic TAG concentrations were determined after enzymatic hydrolysis with lipases using a commercial kit (Randox Laboratories, San Francisco, CA, USA).

#### *Plasma alanine aminotransferase activity*

Plasma alanine aminotransferase activities were measured by catalysing the formation of pyruvate from alanine<sup>(22)</sup>. Enzyme activity was expressed as  $\mu\text{Kat/l}$ .

#### *Quantification of faecal microflora by the fluorescence in situ hybridisation method*

The genotypic probe Bif164 and a non-specific nucleic acid stain, 4',6-diamidino-2-phenylindole, were used to quantify bifidobacteria and total bacteria, respectively<sup>(23)</sup>. Probe fluorescence was detected with a Zeiss Axioskop2 microscope (Carl Zeiss, Jena, Germany), as described previously<sup>(24)</sup>.

#### *Histological evaluation*

Liver tissues were fixed in Bouin's solution overnight and then processed for histological routine. Paraffin sections (4  $\mu\text{m}$ ) were mounted on microscope slides and stained with haematoxylin and eosin. Histological evaluation was done under 200 $\times$  magnification.

#### *Statistical analyses*

Data were presented as means with their standard errors and analysed using SPSS version 14 (SPSS, Inc., Chicago, IL, USA). Parametric data and log-transformed bacteria counts were analysed by one-way ANOVA, followed by the least significant difference test. Differences were considered significant at  $P < 0.05$ .

## **Results**

#### *Body and liver weights*

There were no significant differences in body weight and weight gain among groups (data not shown). Relative liver weight (percentage of body weight) was similar among groups, 4.7 (SE 0.6), 4.7 (SE 0.5), 4.6 (SE 0.4) and 4.7 (SE 0.6)% for the control, DG, DG + FO and DG + vitamin E groups, respectively.

**Table 1.** Hepatic antioxidative enzyme activities in D-galactose-treated Balb/cJ mice(Mean values with their standard errors, *n* 6)

Group	SOD (IU/mg protein)		GPx (IU/mg protein)		Catalase (IU/mg protein)	
	Mean	SE	Mean	SE	Mean	SE
Control	80.0 <sup>a</sup>	5.7	46.2 <sup>a</sup>	4.2	844.7 <sup>a,b</sup>	53.9
DG	68.1 <sup>b</sup>	4.9	32.5 <sup>b</sup>	3.3	726.2 <sup>a</sup>	49.5
DG + FO	78.4 <sup>a</sup>	3.4	41.1 <sup>a,b</sup>	2.0	793.9 <sup>a,b</sup>	41.9
DG + vitamin E	82.4 <sup>a</sup>	3.5	37.5 <sup>a,b</sup>	4.5	871.2 <sup>b</sup>	49.8

SOD, superoxide dismutase; GPx, glutathione peroxidase; DG, D-galactose; FO, fructo-oligosaccharide.

<sup>a,b</sup>Mean values with unlike superscript letters within a column were significantly different ( $P < 0.05$ ; ANOVA followed by the least significant difference test).

### Antioxidative enzyme activities and TAG content in the liver

Superoxide dismutase and glutathione peroxidase activities were significantly suppressed by DG treatment by approximately 32.3% ( $P < 0.001$  *v.* control) and approximately 29.7% ( $P = 0.016$  *v.* control), respectively (Table 1). Both FO ( $P < 0.001$  *v.* DG) and vitamin E ( $P < 0.001$  *v.* DG) reversed the DG-induced decrease in superoxide dismutase activity, and tended to ameliorate ( $P = 0.49$  for DG + FO *v.* control;  $P = 0.11$  for DG + vitamin E *v.* control) the DG-induced decrease in glutathione peroxidase activity. Catalase activity was non-significantly altered by DG ( $P > 0.05$  *v.* control). However, catalase activity in the DG + vitamin E group was raised by approximately 20.0% ( $P = 0.049$  *v.* DG). Hepatic TAG concentration was 234.4 (SE 5.6), 238.6 (SE 3.4) and 243.8 (SE 5.6)  $\mu\text{mol/g}$  liver in the control, DG + FO and DG + vitamin E

groups, respectively, all of which were significantly lower than that shown in the DG group, 252.6 (SE 3.4)  $\mu\text{mol/g}$  liver ( $P < 0.05$ , respectively).

### Plasma alanine aminotransferase activity

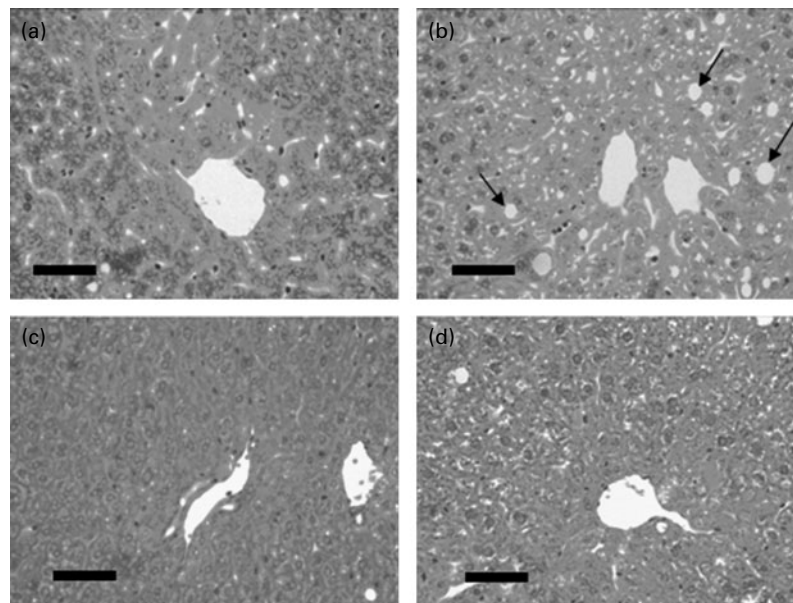
The plasma alanine aminotransferase level ( $\mu\text{Kat/l}$ ) was 0.20 (SE 0.03), 0.32 (SE 0.05) ( $P = 0.016$  *v.* control), 0.22 (SE 0.02) and 0.17 (SE 0.02) in the control, DG, DG + FO and DG + vitamin E groups, respectively. The DG-induced change in alanine aminotransferase activity was normalised by FO ( $P > 0.05$  *v.* control) and vitamin E ( $P > 0.05$  *v.* control), respectively.

### Histopathological observation

The liver histological study was conducted to determine the protective effect of FO on DG-induced injury. The hepatocytes in the DG group were filled with lipids in the absence of the nucleus (Fig. 1(b)). However, this histological alteration was not observed in the control (Fig. 1(a)) group, and was ameliorated in the presence of FO (Fig. 1(c)) and vitamin E (Fig. 1(d)).

### Faecal microflora

The faecal bifidobacteria concentration was the lowest in the DG group, which was significantly increased by FO ( $P = 0.001$  *v.* DG) and vitamin E ( $P = 0.022$  *v.* DG), respectively (Table 2). The faecal total bacteria counts were similar among groups. FO significantly ( $P = 0.032$  *v.* DG) increased the relative proportions (percentage of total bacteria) of



**Fig. 1.** Liver histology in Balb/cJ mice treated for 52 d with (a) vehicle control (saline, subcutaneous (s.c.)), (b) D-galactose (1.2 g/kg, s.c.), (c) D-galactose (1.2 g/kg, s.c.) + fructo-oligosaccharide (5%, w/w) or (d) D-galactose + vitamin E (0.2%, w/w). Livers were dissected after systematic perfusion with neutral formalin (*n* 4) for 5 min and then fixed in Bouin's solution overnight. Tissues were processed for histological routine and stained with haematoxylin and eosin (original magnification, 200 $\times$ ). Scale bar represents 50  $\mu\text{m}$ .

**Table 2.** Faecal total bacteria and *Bifidobacterium* counts of D-galactose-treated Balb/cJ mice(Mean values with their standard errors, *n* 10)

	<i>Bifidobacterium</i> (log <sub>10</sub> counts/g dry faeces)		Total bacteria (log <sub>10</sub> counts/g dry faeces)	
	Mean	SE	Mean	SE
Control	6.65 <sup>b</sup>	0.02	7.25	0.03
DG	6.57 <sup>a</sup>	0.02	7.26	0.04
DG + FO	6.73 <sup>c</sup>	0.02	7.22	0.02
DG + vitamin E	6.66 <sup>b</sup>	0.02	7.22	0.03

FO, fructo-oligosaccharide; DG, D-galactose.

<sup>a,b,c</sup> Mean values with unlike superscript letters within a column were significantly different (*P* < 0.05; ANOVA followed by the least significant difference test).

faecal bifidobacteria to 33.0 (SE 2.3)% compared with that in the DG group, 21.0 (SE 2.0)%.

## Discussion

This is the first study to show that FO, besides its prebiotic effect on colonic microflora<sup>(13,14)</sup>, exerted systematic effects on antioxidative enzyme activities and TAG synthesis in the liver that were altered by chronic DG administration in Balb/cJ mice.

D-Galactose is normally metabolised by D-galactokinase and galactose-1-phosphate uridylyltransferase<sup>(25)</sup>. An overdose of DG leads to the accumulation of galactitol, which in turn leads to osmotic stress and the generation of reactive oxygen species<sup>(26)</sup>. In the present study, we found that FO, similar to the antioxidant vitamin E, prevented the decrease in hepatic superoxide dismutase activity and potentially modulated glutathione peroxidase activity, suggesting that the utilisation of FO in the large intestine exerted systematic antioxidative effects. Although mechanisms remained unclear, *in vitro* studies have shown that lactic acid bacteria *per se*<sup>(27,28)</sup> and the fermentations of FO by several strains of bifidobacteria<sup>(29)</sup> exert free radical-eliminating effects. Therefore, FO may reduce hepatic oxidative stress partially through its fermentation product by colonic lactic acid bacteria. An increased faecal bifidobacteria concentration observed in the DG + FO group could further enhance the antioxidative ability of FO.

Incorporation of FO into a high-carbohydrate diet has been shown to suppress the activity of lipogenic enzymes in rats<sup>(30)</sup>. The present study further indicated that FO reduced fatty liver in DG-treated mice, which may be mediated by propionate that is shown to down-regulate liver lipogenesis<sup>(31)</sup>. Vitamin E diminished hepatic TAG accumulation in the present study, which, on the other hand, may be mediated by its potential enhancing effect on PPAR<sup>(32)</sup> that is shown to regulate fatty acid oxidation<sup>(33)</sup>.

FO is easily incorporated into drinks. The elderly with poor oral function can obtain sufficient dietary fibre by taking this type of dietary supplement. The level of FO

offered in the present study is equivalent to 25 g/d for adults whose daily dry food intake is 500 g. An adequate intake for total fibre in foods is set as 25 and 38 g/d for young women and men, respectively<sup>(34)</sup>. Therefore, the dose of FO supplement used in the present study is applicable to adults, including the elderly.

In conclusion, the present study suggests that FO, besides being a prebiotic fibre, could prevent oxidative stress and fatty liver that occur during ageing.

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