Effects of Lotus Root (the Edible Rhizome of *Nelumbo nucifera*) on the Development of Non-Alcoholic Fatty Liver Disease in Obese Diabetic db/db Mice

Yumi Tsuruta,1,2,3 Koji Nagao,1,2 Bungo Shirouchi,1 Saori Nomura,1 Keisuke Tsuge,3 Kazuyoshi Koganemaru,3 and Teruyoshi Yanagita1,2,4

1Department of Applied Biochemistry and Food Science, Saga University, Saga 840-8502, Japan
2Department of Biochemistry and Applied Biosciences, United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima 890-8580, Japan
3Industrial Technology Center of Saga, Saga 849-0932, Japan

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Non-alcoholic fatty liver disease (NAFLD) is emerging as the most common liver disease in industrialized countries. The discovery of food components that would ameliorate NAFLD is therefore of interest. Lotus root, the edible rhizome of *Nelumbo nucifera*, contains a high level of polyphenolic compounds, and several health-promoting properties of lotus root have been reported. The present study examines whether dietary lotus root powder can protect db/db mice from hepatic injury. After 3 weeks of feeding, the hepatomegaly, hepatic triglyceride accumulation, and elevated hepatic injury markers in the serum were markedly alleviated in the Lotus diet-fed db/db mice relative to the control mice. These effects were partly attributable to suppression of the lipogenic enzyme activities and mRNA expression by the Lotus diet. The serum levels of adiponectin, which has been reported to have a protective effect against NAFLD, were significantly higher in the Lotus group mice. Moreover, the hepatic expression of such inflammatory genes as tumor necrosis factor-alpha and monocyte chemoattractant protein-1 were markedly suppressed by the Lotus diet. We speculate that the development and progression of NAFLD were prevented by suppressing the expression of lipogenic and inflammatory genes as a result of the higher serum adiponectin level in the Lotus diet-fed db/db mice.

Key words: adiponectin; db/db mice; Lotus root; metabolic syndrome; non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is often associated with features of metabolic syndrome and is emerging as the most common liver disease worldwide.1–4) NAFLD is the preferred term used to describe the spectrum of liver damage ranging from hepatic steatosis to steatohepatitis, liver fibrosis and cirrhosis. Most liver-related morbidity and mortality is associated with the development of cirrhosis which is most likely to occur in individuals who have progressed from hepatic steatosis to steatohepatitis. The process by which steatohepatitis evolves from hepatic steatosis is not fully understood; nevertheless, it is necessary to develop effective therapies for treating NAFLD, and the discovery of nutrients that will reduce the risk of NAFLD would be useful. db/db Mice suffer from hyperphagia, because they have a mis-sense mutation on the leptin receptor gene, and develop a syndrome involving multiple metabolic and hormonal disorders, including NAFLD which shares many features with human metabolic syndrome.5–7)

Diet has been recognized as a factor contributing to the development and prevention of NAFLD,8–11) and polyphenol-rich plants and fruits have been used in folk medicine for treating life-style related diseases throughout the world.12–14) *Nelumbo nucifera* is a plant in the monogeneric family of *Nelumbonaceae*, whose rhizome (lotus root) is recognized in eastern countries as one of the most delicious and nutritional vegetables that has also been used in traditional Asian herbal medicine. Previous studies have shown that lotus root contains a high level of polyphenolic compounds and possesses such health-beneficial properties as hypoglycemic, anti-inflammatory and antioxidative activities.15–18) The effects of lotus root on lipid metabolism, however, have not been fully studied. We evaluated in the present study the effect of a lotus root powder-supplemented diet on the development of NAFLD in db/db mice.

Materials and Methods

*Animals and diets.* All aspects of the experiment were conducted according to the guidelines provided by the ethical committee for experimental animal care at Saga University. Five-week-old male db/db mice were purchased from Japan SLC (Shizuoka, Japan). The mice were individually housed in plastic cages in a temperature-controlled room (24 °C) under a 12-h light/dark cycle. The basal semi-synthetic diets were prepared according to the recommendations of AIN-7619) (Table 1). The freeze-dried lotus root powder was provided by the Industrial Technology Center of Saga, and the amounts of the general components in the samples were routinely determined according to AOAC official methods. The amount of total polyphenols was

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1 To whom correspondence should be addressed. Tel/Fax: +81-952-28-8782; E-mail: yanagitt@cc.saga-u.ac.jp

Abbreviations: ACC1, acetyl-CoA carboxylase-1; ALP, alkaline phosphatase; CPT, carnitine palmitoyltransferase; CRP, c-reactive protein; FAS, fatty acid synthase; GPT, glutamic pyruvic transaminase; MCP1, monocyte chemoattractant protein-1; TNF alpha, tumor necrosis factor-alpha; WAT, white adipose tissue; NAFLD, non-alcoholic fatty liver disease
Table 1. Composition of Experimental Diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control (g/kg)</th>
<th>Lotus (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200.00</td>
<td>195.10</td>
</tr>
<tr>
<td>Corn starch</td>
<td>150.00</td>
<td>117.30</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.00</td>
<td>42.75</td>
</tr>
<tr>
<td>Mineral mixture (AIN 76)</td>
<td>35.00</td>
<td>35.00</td>
</tr>
<tr>
<td>Vitamin mixture (AIN 76)</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>n-3-Methionine</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Corn oil</td>
<td>50.00</td>
<td>50.00</td>
</tr>
<tr>
<td>Lotus root powder-a</td>
<td>0.00</td>
<td>50.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>500.00</td>
<td>494.85</td>
</tr>
</tbody>
</table>

a Carbohydrate, 65.4%; fiber, 14.5%; protein, 9.8%; ash, 5.8%; water, 4.1%; fat, 0.4%.
b Total polyphenols: 11.9 mg/g (gallic acid equivalent).

determined by using gallic acid as a standard equivalent according to the Folin-Ciocalteu method. The db/db mice were assigned to two groups of six mice each that were fed one of the two diets (Table 1): a semisynthetic AIN-76 diet (Control group) or a semisynthetic AIN-76 diet supplemented with 5% dried lotus root powder (Lotus group). Since lotus root powder contained carbohydrate (65.4%), rich in starch, fiber (14.5%), protein (9.8%), and other components, it was supplemented to the diet at the expense of corn starch, cellulose, casein, and sucrose (Table 1). The mice were pair-fed with the diets using Rodent CAFE (KBT Oriental Co., Saga, Japan) for 3 weeks. At the end of the feeding period, the mice were sacrificed by exsanguination from the heart under pentobarbital sodium salt anesthesia after a 9-h starvation period. White adipose tissue (WAT) and the liver were immediately excised, and the serum was separated from the blood.

Measurement of the triglyceride and cholesterol levels in the liver. Liver lipids were extracted according to the method of Folch et al. and the concentrations of triglyceride and cholesterol were respectively measured by using the methods of Fletcher and Sperry and Webb.

Measurement of the serum parameters. The serum triglyceride and cholesterol levels were measured by using commercial enzyme assay kits (Wako Pure Chemicals, Tokyo, Japan). The activities of glutamic pyruvic transaminase (GPT) and alkaline phosphatase (ALP) in the serum were also measured by using commercial enzyme assay kits (Wako Pure Chemicals, Tokyo, Japan). The serum adiponectin level was measured by using a commercial mouse ELISA kit (Otsuka Pharmaceutical Co., Tokyo, Japan).

Assays of the hepatic enzyme activity. The enzyme activities of fatty acid synthase (FAS) and the malic enzyme in the cytosol fraction and of carnitine palmitoyltransferase (CPT) in the mitochondrial fraction were determined as described elsewhere. The protein concentration of each fraction was determined according to the method of Lowry et al. with bovine serum albumin used as the standard.

Analysis of mRNA expression. Total RNA was extracted from 100 mg of liver by using an RNAsafe Lipid Tissue mini kit (Qagen, Tokyo, Japan). The TaqMan Universal PCR Master mix (Applied Biosystems, Tokyo, Japan) and Assay-on-Demand gene expression products (Mm01304289m1 for acetyl-CoA carboxylase-1 [ACC1], Mm0066239_m1 for FAS, Mm00432680_m1 for c-reactive protein [CRP], Mm00414124_m1 for monocyte chemoattractant protein-1 [MCP1], Mm0043258_m1 for tumor necrosis factor-alpha [TNF alpha], and Hs99999901_s1 for 18S RNA; all from Applied Biosystems, Tokyo, Japan) were respectively used for the quantitative real-time RT-PCR analysis of ACC1, FAS, CRP, MCP1, TNF alpha and 18S RNA expression in the liver. Amplification was carried out by using an ABI Prism 7000 real-time PCR sequence detection system (Applied Biosystems).

Statistical analysis. Each data value is expressed as the mean ± standard error. The significance of differences between the means for the two groups was determined by Student’s t-test, differences being considered significant at p < 0.05.

Results

The two groups of db/db mice did not differ in their initial body weight, nor did they differ in their final body weight, food intake, or total WAT weight during and after the 3-week feeding period (Table 2). In contrast, the appearance of the liver, relative liver weight and hepatic triglyceride concentration all differed between the db/db mice fed with the Control and Lotus diets (Fig. 1). The relative liver weight was 25% less in the Lotus diet-fed db/db mice, and this lower liver weight was associated with markedly less (64%) triglyceride accumulation in the liver (Fig. 1). However, the hepatic cholesterol level was no different between the two groups (Control group, 2.37 ± 0.14 mg/g of liver; Lotus group, 2.93 ± 0.27 mg/g of liver). The extent and direction (increase or decrease) of changes in the liver lipoprotein synthesis and secretion during the development of NAFLD have been controversial. The serum cholesterol levels were no different in our study (Control group, 236 ± 17 mg/dL; Lotus group, 218 ± 16 mg/dL), but the serum triglyceride levels were higher (Control group, 36.2 ± 3.9 mg/dL; Lotus group, 66.0 ± 9.8 mg/dL, p < 0.05) in the Lotus group than in the

Table 2. Effect of Lotus Root Powder on Growth Parameters in db/db Mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Lotus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight</td>
<td>27.2 ± 0.5</td>
<td>27.2 ± 0.4</td>
</tr>
<tr>
<td>Final body weight</td>
<td>35.9 ± 0.7</td>
<td>32.7 ± 1.5</td>
</tr>
<tr>
<td>Food intake</td>
<td>110 ± 3</td>
<td>110 ± 1</td>
</tr>
<tr>
<td>White adipose tissue</td>
<td>9.82 ± 0.22</td>
<td>9.71 ± 0.16</td>
</tr>
<tr>
<td>Total</td>
<td>4.51 ± 0.16</td>
<td>4.66 ± 0.16</td>
</tr>
<tr>
<td>Perirenal</td>
<td>1.81 ± 0.12</td>
<td>1.68 ± 0.13</td>
</tr>
<tr>
<td>Omental</td>
<td>3.51 ± 0.12</td>
<td>3.38 ± 0.14</td>
</tr>
</tbody>
</table>

Each value is expressed as the mean ± standard error for six mice.

Fig. 1. Appearance of the Liver, Relative Liver Weight and Hepatic Triglyceride Level in the db/db Mice.

The mice were fed with the Control diet or Lotus root powder diet for 3 weeks. Each value is expressed as the mean ± standard error for six mice. See Table 1 for the composition of each diet. *Significant difference at p < 0.05 between the Control and the Lotus groups of db/db mice.
Control group. Consistent with the alleviation of hepatomegaly and hepatic steatosis by the Lotus diet, the activities of such hepatic injury markers as GPT and ALP were markedly lower (by 31% and 42%, respectively) in the sera of the Lotus diet-fed db/db mice than in the sera of the Control diet-fed db/db mice (Fig. 2). The serum level of adiponectin, which has been reported to have a protective effect against NAFLD, was significantly higher in the Lotus group than in the Control group (Fig. 3). These results suggest that the higher serum adiponectin level may have contributed to preventing the development of NAFLD in the db/db mice fed with the Lotus diet.

To further examine the effect of the Lotus diet on the liver, the hepatic enzymes related to triglyceride metabolism were analyzed (Table 3). Although the activity of CPT, a key enzyme in fatty acid β-oxidation, remained unchanged, the activities of FAS and the malic enzyme, which are lipogenic enzymes related to de novo fatty acid biosynthesis, were significantly lower in the Lotus diet-fed db/db mice than in the Control diet-fed db/db mice. These results suggest that the alleviation of NAFLD by the Lotus diet was partially attributable to suppression of the lipogenic enzyme activities in the liver.

To gain insight into the effect of the Lotus diet on the levels of mRNA as related to the development and progression of NAFLD, we analyzed the hepatic mRNA expression of lipogenic and inflammatory genes by quantitative real-time RT-PCR (Fig. 4). Consistent with suppression of the lipogenic enzyme activities by the Lotus diet, the ACC1 mRNA level was significantly lower (by 39%) and the FAS mRNA level tended to be lower (by 38%) in the liver of the Lotus-diet-fed db/db mice than in those fed with the Control diet (Fig. 4). Additionally, the expression levels of such inflammatory genes as MCP1 and TNF alpha, but not CRP, were markedly lower (by 64% and 52%, respectively) in the liver of the Lotus-diet-fed db/db mice than in the liver of the mice fed with the Control diet (Fig. 4).

**Discussion**

NAFLD is common in type 2 diabetic and obese patients. Although the mechanisms responsible for the development of NAFLD are unclear, it has been suggested that hepatic steatosis results from increased lipogenesis and/or decreased lipolysis, in addition to the accelerated mobilization of fat from expanded visceral WAT to the liver. The Lotus diet used in the present study counteracted both the hepatomegaly and hepatic steatosis that occur in obese db/db mice. The alleviation of NAFLD by the Lotus diet was partially attributable to suppression of the lipogenic enzyme activities in the liver. Although we did not carry out a histological evaluation of the liver, our data indicate that the Lotus
diet protected \(db/db\) mice from the development of NAFLD.

It has recently been recognized that adipose tissue stores excess energy in the form of fat and also secretes physiologically active substances called adipokines.\(^{31}\) Among these adipokines, adiponectin is one of the most abundant secretory proteins produced by the adipose tissue in rodents and humans.\(^{31,32}\) Previous studies have indicated that adiponectin had a protective effect against NAFLD.\(^{28–30}\) The serum adiponectin level in the present study was significantly higher in the Lotus group than that in the Control group of \(db/db\) mice (Fig. 3). A previous report has also suggested that adiponectin suppressed hepatic lipogenesis through the down-regulation of SREBP 1c, the master regulator that controls the enzymes involved fatty acid synthesis.\(^{32}\)

The mRNA expression levels of such lipogenic genes as ACC1 and FAS in the present study were lower in the \(db/db\) mice from the development of \(db/db\) mice. Since it has recently been recognized that adipose tissue characterizes the active components of the Lotus Root Alleviates NAFLD in \(db/db\) Mice

The pathogenesis of steatohepatitis, the more advanced form of NAFLD, has yet to be clearly defined, but the major theory recently proposed is the “two-hit” hypothesis.\(^{33}\) The first “hit” is triglyceride accumulation within the liver. It has been proposed that lipid-laden hepatocytes are more susceptible to a second “hit,” i.e., injury as the result of oxidative stress and such inflammatory cytokines as TNF alpha. In addition, lipid peroxidation products trigger cytokine production within the liver, and this would accelerate the TNF alpha-mediated liver injury. It is a fact that over-expression of TNF alpha mRNA occurs in the liver of a non-alcoholic steatohepatitis patient.\(^{34}\) It has also been recognized that MCP1, a member of the CC chemokine family, induced inflammatory responses through the recruitment of inflammatory cells and was up-regulated by such inflammatory stimuli as TNF alpha.\(^{35,36}\) Recent findings have shown that MCP1 overexpression induced hepatic steatosis, whereas MCP1 deletion improved hepatic steatosis in genetically-manipulated mice.\(^{37}\) It has also been suggested that obesity enhanced the synthesis of CRP, an inflammatory cytokine, and an elevated CRP mRNA level in the present study remained unchanged, whereas MCP1 deletion improved hepatic steatosis in genetically-manipulated mice.\(^{37}\) It has also been suggested that obesity enhanced the synthesis of CRP, an inflammatory cytokine, and an elevated CRP mRNA level in the present study remained unchanged, whereas MCP1 deletion improved hepatic steatosis in genetically-manipulated mice.\(^{37}\)

In conclusion, our present results suggest that the suppressed hepatic expression of lipogenic and inflammatory genes prevented the development and progression of NAFLD through enhancement of the serum adiponectin level in the Lotus diet-fed \(db/db\) mice.

Acknowledgments

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References

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